IRON-RELATED PARAMETERS IN ADIPOSE TISSUE AND BLOOD IN DIABETES MELLITUS

DISSERTATION

Submitted to

THE TAMILNADU DR.MGR MEDICAL UNIVERSITY

In partial fulfillment for the degree

DOCTOR OF MEDICINE

IN

BIOCHEMISTRY - BRANCH XIII

MAY 2018

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DEPARTMENT OF BIOCHEMISTRY

CHRISTIAN MEDICAL COLLEGE

VELLORE-632002, INDIA

CERTIFICATE

This is to certify that the study titled **"IRON-RELATED PARAMETERS IN ADIPOSE TISSUE AND BLOOD IN DIABETES MELLITUS"** is the bona fide work of Dr. Rosa Mariam Mathew, who conducted it under the guidance and supervision of Dr. Molly Jacob, Professor of Biochemistry, Christian Medical College, Vellore. The work in this dissertation has not been submitted to any other university for the award of a degree.

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DECLARATION

I hereby declare that the investigations, which form the subject matter of this study, were conducted by me under the supervision of Dr. Molly Jacob, Professor of Biochemistry, Christian Medical College, Vellore.

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PLAGIARISM CHECK

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ABSTRACT

Iron-related parameters in adipose tissue and blood in diabetes mellitus

Introduction

Type 2 diabetes mellitus (T2DM) has been shown to be associated with increased body iron stores. The iron content in adipose tissue has been postulated to play a role in the pathogenesis of insulin resistance, a characteristic feature of T2DM.

Aim

To study iron-related parameters in adipose tissue and blood in T2DM patients and to compare them with control subjects

Objectives

1. To determine mRNA expression of transferrin receptor 1 (TfR1) (the iron import protein) and ferroportin (the iron export protein) in subcutaneous and visceral adipose tissue in patients with T2DM and in control subjects
2. To compare serum levels of iron, ferritin and transferrin saturation in patients with T2DM and control subjects
3. To obtain anthropometric data of these patients and correlate these with the above parameters

Materials and Methods

Patients who underwent elective abdominal surgery were the subjects of this study. Such patients were classified as diabetics or controls. Anthropometric data and blood samples were collected from the patients preoperatively. Blood was used to estimate various iron-related parameters. Samples of sub-cutaneous and visceral adipose tissue were collected at the time of surgery. These samples were used to determine gene expression of ferroportin and TfR1.

Results

Twenty three diabetics and 14 control subjects were studied. Blood parameters of iron status and body mass index were similar in both groups. TfR1 mRNA levels tended to be higher in the visceral adipose tissue of the diabetic group compared to controls (p value = 0.069). There was a significant correlation between TfR1 and ferroportin mRNA levels in the visceral adipose tissue of diabetics.

Conclusion

The observations of this study suggest that adipocytes in VAT from diabetics may be iron-depleted, as indicated by a trend for TfR1 mRNA levels to be higher in diabetics. This observation requires confirmation in an adequate sample size of patients.

Keywords: Type 2 diabetes mellitus, insulin resistance, adipose tissue, transferrin receptor 1, ferroportin

REVIEW OF LITERATURE

DIABETES MELLITUS

Diabetes mellitus (DM) is a state of metabolic dysregulation characterized by hyperglycemia (Kasper et al., 2015). The metabolic dysregulation associated with DM leads to secondary pathophysiologic changes in multiple organ systems, causing increased levels of DM-related morbidity and mortality (Roglic, 2016). According to the mortality database of the World Health Organization (WHO), DM has become the eighth leading cause of death among both sexes and the fifth leading cause of death in women (Roglic, 2016). The global prevalence of diabetes among the adult population has almost doubled since 1980, rising from 4.7% to 8.5% (Roglic, 2016). The potential complications of diabetes are enormous, causing significant healthcare burdens on both families and society (International Diabetes Federation, 2015). As a developing country, India should be more aware about non-communicable diseases like DM, hypertension and coronary artery diseases, which seriously impact the health of the country's population. Indian reports have shown that more than 62 million people in India have been diagnosed to have DM (Joshi and Parikh, 2007; Kumar et al., 2013). In the state of Tamil Nadu, 9.8 per cent of the state's population (42 lakh people) is living with the disease. Tamil Nadu has the highest number of diabetics in the country. It also showed that 3 million people in the state are at high-risk of developing diabetes (Anjana, 2011).

Classification of DM

DM is classified based on the pathogenic process that leads to hyperglycemia. The two major categories of DM are type 1(T1DM) and type 2 (T2DM) (Kasper et al., 2015). Other types of DM include gestational diabetes (GDM) and other specific types, which include monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young [MODY]), diseases of the exocrine pancreas, endocrinopathies, drug related etc (Kasper et al., 2015). Each specific type of DM is diagnosed based on established criteria (Thomas et al., 2016).

Deficiency of insulin is the basic characteristic of T1DM. But T2DM is a heterogeneous group of disorders with variable degrees of insulin resistance, impaired insulin secretion and increased hepatic glucose production (Kasper et al., 2015).

Type 2 diabetes mellitus (T2DM)

Insulin resistance and abnormal insulin secretion play the key roles in the pathology of T2DM (Kasper et al., 2015). A subnormal biological response to both endogenous and exogenous insulin is known as insulin resistance (Mantzoros et al., 2016; Moller and Flier, 1991). Insulin resistance has a very broad spectrum of clinical presentations, with classical presentation being elevated blood glucose levels in spite of the large doses of insulin administration (Mantzoros et al., 2016). Other clinical features that suggest insulin resistance include acanthosis nigricans, ovarian hyperandrogenism (polycystic ovary syndrome [PCOS]), lipodystrophy, accelerated or impaired linear growth, autoimmunity and muscle cramps (Mantzoros et al., 2016). Both genetic and environmental factors play an important role in the development of insulin resistance and T2DM. The age of onset in T2DM is usually late, between 40 and 59 years. For example, the *TCF7L2* gene has found to have a strong association with T2DM (Kasper et al., 2015). Environmental factors associated with T2DM

include ageing, central obesity, unhealthy dietary habits, sedentary life styles, economic development and increasing urbanisation (Kasper et al., 2015; Roglic and World Health Organization, 2016). Among the different risk factors that contribute to the development of T2DM, obesity is found to have a very significant role and more than 90% of type 2 diabetics are overweight or obese (WHO, 2016)

Diagnosis of DM

The following are criteria of the American Diabetes Association (ADA) (2015) for the diagnosis of DM.

"A hemoglobin A1c (HbA1c) level of 6.5% or higher **or**

A fasting plasma glucose (FBS) level of 126 mg/dL (7 mmol/L) or higher; fasting is defined as no caloric intake for at least 8 hours, **or**

A 2-hour plasma glucose level of 200 mg/dL (11.1 mmol/L) or higher during a 75-g oral glucose tolerance test (OGTT), **or**

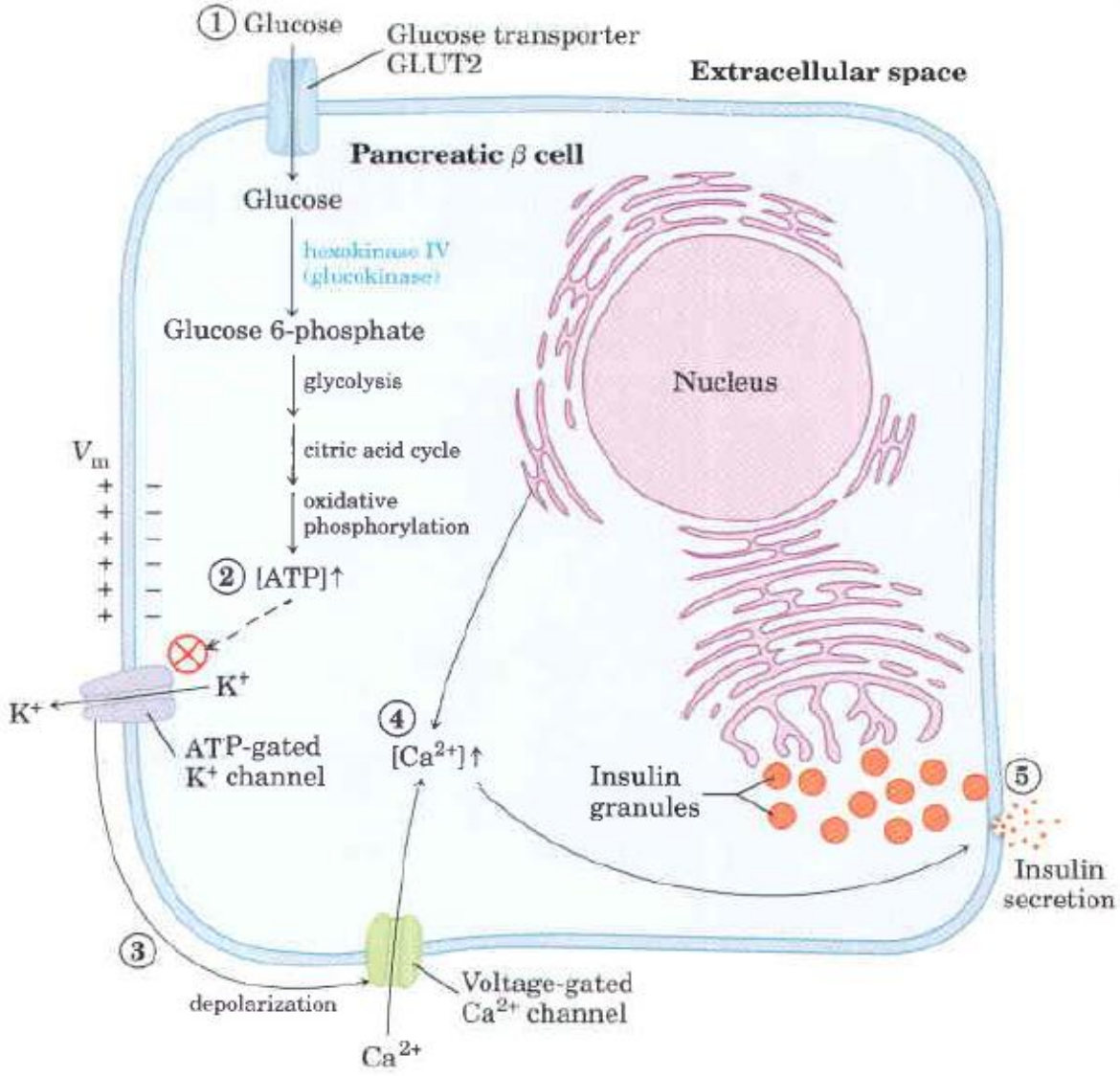
A random plasma glucose (RBS) of 200 mg/dL (11.1 mmol/L) or higher in a patient with classic symptoms of hyperglycemia (i.e., polyuria, polydipsia, polyphagia, weight loss)"

INSULIN

Insulin, a 51-amino acid peptide hormone, helps in the cellular uptake and utilization of glucose (Rodwell et al., 2015). It is secreted by the beta-cells of pancreas as an 86-amino-acid precursor polypeptide, preproinsulin. The amino-terminal signal peptide is removed on subsequent proteolytic processing that gives rise to proinsulin (Rodwell et al., 2015). Further removal of a 31-residue fragment from proinsulin leads to the formation of the C-peptide and the A and B chains of insulin (Kasper et al., 2015). The A chain (with 21 amino acids) and the B chain (with 30 amino acids) are linked by disulfide bonds (Rodwell et al., 2015). The mature insulin peptide hormone and the C-peptide are stored together in the secretory granules in the beta-cells. At the time of secretion of the hormone, insulin and the C-peptide are co-secreted, which makes the C- peptide a useful marker of insulin secretion, as the C-peptide is cleared more slowly than insulin (Kasper et al., 2015).

The key regulator of insulin secretion is glucose (Rodwell et al., 2015). Insulin synthesis, especially protein translation and processing, is enhanced by glucose levels that exceed 3.9 mmol/L (70 mg/dL) (Kasper et al., 2015). Glucose is transported into the beta-cells by a facilitative glucose transporter (GLUT2) (Kasper et al., 2015). Glucose is then phosphorylated by glucokinase, which is the rate-limiting step of glucose-regulated insulin secretion. The glucose-6-phosphate formed generates ATP via glycolysis. Generation of ATP results in the inhibition of the activity of an ATP-sensitive K^+ channel, which induces depolarization of the membrane of the beta-cell. This opens voltage-dependent calcium channels, leading to an influx of calcium and thus stimulating insulin secretion (Kasper et al., 2015). Insulin secretion has a pulsatile pattern of hormone release (Kasper et al., 2015).

Figure 2.1. Insulin secretion triggered by glucose



Lehninger's Principles of Biochemistry 6th edition, 2013

Fifty percent of insulin secreted is removed and degraded by the liver, once it is secreted into the portal venous system (Kasper et al., 2015). The un-extracted fraction of insulin enters the systemic circulation. It binds to its receptors on cells at target sites. The insulin receptor is a tyrosine kinase receptor (Rodwell et al., 2015). Binding of insulin to its receptor leads to intrinsic tyrosine kinase activity, followed by autophosphorylation of the receptor and subsequent recruitment of insulin receptor substrates (IRS), which are intracellular signalling molecules (Rodwell et al., 2015). This is followed by a complex cascade of phosphorylation and dephosphorylation reactions, ending in the metabolic and mitogenic effects of insulin, through different pathways which includes Ras and mitogen-activated protein (MAP) kinase pathway, phosphatidylinositol 3 kinase (PI3- kinase) pathway and also through phospholipase C γ (Lieberman et al., 2013). The PI 3-kinase pathway results in the activation of protein kinase B (also called Akt), which is a serine–threonine kinase.

Activation of the Akt pathways leads many of the downstream effects of insulin on glucose metabolism (Lieberman et al., 2013). Insulin promotes the translocation of GLUT4 (glucose transporter 4) to the cell surface, which helps in the uptake of glucose by skeletal muscle and adipose tissue. It also induces glucokinase and glycolysis, thus helping in the utilisation of glucose. Insulin is an anabolic hormone and it promotes glycogen synthesis, protein synthesis, and also lipogenesis. In addition to the above metabolic effects, insulin also inhibits gluconeogenesis and glycogenolysis, thus preventing elevation of glucose levels in the blood (Lieberman et al., 2013; Rodwell et al., 2015).

Insulin is the main regulator of glucose homeostasis, which is basically the balance between glucose production by the liver and peripheral glucose uptake and utilization (Kasper et al., 2015). Deficiency of insulin or inadequate ability of the cells to respond to insulin thus leads to metabolic dysregulation and related complications. Acute complications of DM include

diabetic ketoacidosis or hyperosmolar nonketotic coma (Kasper et al., 2015). Chronic complications of DM are quite common and are broadly classified in to macro and microvascular complications (Kasper et al., 2015). Macrovascular complications lead to coronary artery disease, cerebrovascular diseases, and peripheral arterial disease. Microvascular complications include retinopathy, neuropathy and nephropathy. Repeated infections, cataract, gastrointestinal and genitourinary problems and dermatologic problems are also associated with DM (Kasper et al., 2015).

OBESITY AND TYPE 2 DM

T2DM is usually associated with obesity, particularly of the central (visceral) type (Kasper et al., 2015). More than 80 percent of cases of T2DM can be attributed to obesity (Bray and Perreault, 2017). The risk of type 2 diabetes rises with the increase in body weight (Helmrich et al., 1991; Mokdad et al., 2003; Nguyen et al., 2011). Obesity is now considered a global epidemic. The prevalence of obesity in adults, adolescents, and children is on the increasing (Bray and Perreault, 2017). In addition to T2DM, obesity is also associated with hypertension, dyslipidemia, heart disease, stroke, sleep apnea, and cancer (Finkelstein et al., 2009).

Obesity is a chronic pathologic condition with abnormal or excessive fat accumulation in the body, which can impair health of a person. The fundamental cause of overweight and obesity is an energy imbalance between calories expended and calories consumed.(WHO, 2016) The surplus energy in the body is stored in adipocytes, leading to adipose tissue expansion, due to hypertrophy or hyperplasia of adipocytes (Murri et al., 2014).

Definitions of overweight and obesity

A weight above "normal" range is referred to be overweight, with normal defined on the basis of actuarial data. This is determined by calculating the body mass index (BMI) (Bray and Perreault, 2017). BMI is defined as the weight in kilograms divided by height in meters squared. Based on BMI values, overweight is defined as a BMI of 25 to 29.9 kg/m^2; obesity is defined as a BMI of ≥30 kg/m^2. Severe obesity is defined as a BMI ≥40 kg/m^2 or ≥35 kg/m^2 in the presence of comorbidities (Bray and Perreault, 2017).

The BMI can be correlated with percentage of body fat mass (Gallagher et al., 1996). It gives a better idea of total body fat than body weight alone (Mei et al., 2002). It may, however, overestimate the degree of fatness in individuals, such a professional athletes or body builders, who are overweight but very muscular. It also gives underestimates in older persons because of the loss of muscle mass associated with aging (Bray and Perreault, 2017).

Screening for overweight and obesity

Screening for overweight and obesity can be done by calculating body mass index (BMI), as already stated. Abdominal adiposity may not be reflected in the BMI range of 25 to 35 $kg/m^{2.}$ Waist circumference is often used as a marker of central obesity. (Bray and Perreault, 2017).

Waist circumference

A waist circumference of ≥40 in (102 cm) for men and ≥35 in (88 cm) for women is considered abnormally high. It is predictive of increased cardiometabolic risk (Jensen et al., 2014). Almost all individuals with BMI ≥35 kg/m^2 have an abnormal waist circumference and are at a high risk from their adiposity; hence, waist circumference measurement does not provide any additional information in these patients (Bray and Perreault, 2017). It is of

particular in use among people whose BMI is in the range of 25 to 35 kg/m^2, where the abdominal adiposity may not be identified easily(Bray and Perreault, 2017).

The waist-to-hip ratio is another parameter frequently used by clinicians; this provides no particular advantage over the waist circumference alone (Bray and Perreault, 2017). It is not currently recommended by the American Heart Association (AHA)/American College of Cardiology (ACC)/The Obesity Society (TOS), as part of the routine evaluation for obesity.

THE ADIPOSE TISSUE

The energy imbalance between calories consumed and calories expended leads to storage of energy in the form of lipid droplet inside adipocytes. These lipid-filled mature adipocytes, along with stromal and vascular fraction of heterogeneous population of cells such as pre-adipocytes, macrophages, endothelial and blood cells, constitute the adipose tissue (Gil et al., 2011). Adipose tissue has two main functions. Regulation of the energy storage by buffering excess triglycerides and free fatty acids is one of them. In addition, it also has endocrine functions. It secretes and regulates numerous hormones and adipokines (Ahima and Flier, 2000), (Kershaw and Flier, 2004).

Types of adipose tissue

Two types of adipose tissue are found in mammals - white and brown adipose tissue. The white adipose tissue is an energy storage tissue that releases triglycerides and free fatty acids during fasting states or situations of energy demand (Murri et al., 2014). Brown adipose tissue plays a major role in the homeostasis of body temperature, by functioning as an energy-dissipating thermogenic organ. Brown adipose tissue is very rich in mitochondria (Murri et al., 2014).

White adipose tissue is seen around viscera as well as under the skin. That which is found around viscera is called visceral adipose tissue (VAT); white adipose tissue that is seen in under the skin is called subcutaneous adipose tissue (SAT) (Murri et al., 2014). VAT is metabolically more active than SAT, with a greater capacity to generate free fatty acids and to take up glucose. VAT adipocytes have been shown to become insulin-resistant to a greater extent than SAT adipocytes, in diabetes mellitus (Wilcox, 2005).

Inflammation of adipose tissue in diabetes mellitus

It is known that inflammation in adipose tissue plays a major role in the metabolic consequences associated with obesity. Such chronic inflammation in obese states plays a major role in the pathogenesis of insulin resistance (de Luca and Olefsky, 2008; Ota, 2013; Shoelson et al., 2006; Xu et al., 2003). The development of obesity is often associated with infiltration of adipose tissue with pro-inflammatory macrophages, resulting in increased secretion of pro-atherogenic, pro-inflammatory and pro-diabetogenic adipokines (resistin, interleukin-6, tumor necrosis factor alpha [TNF-α]), and decreased production of the anti-inflammatory and anti-diabetic adipokine, adiponectin (Murri et al., 2014). In a state of insulin resistance, the action of insulin on insulin-responsive tissues, such as the liver, skeletal muscle and adipose tissue, is impaired by pro-inflammatory metabolic stress (de Luca and Olefsky, 2008). Rates of lipolysis are increased in inflamed adipose tissue, resulting in increased production of free fatty acids (FFA) (Duncan et al., 2007). Adipocyte-derived FFA also stimulate macrophages to produce TNF-α which, in turn, causes further activation of macrophages and differentiation of monocytes into macrophages, thus constituting a vicious cycle (Murri et al., 2014).

T2DM, adipose tissue and iron

Metabolism in adipose tissue in patients with T2DM has been shown to be affected by both iron deficiency and excess (Fernández-Real et al., 2015). The iron content of adipose tissue has been shown to affect whole-body insulin sensitivity (J. M. Moreno-Navarrete et al., 2014).

IRON

Iron, the second most abundant metal on earth is an essential micronutrient in the human body. It plays important roles in oxygen transport, regulation of cell growth and differentiation, mitochondrial respiration, DNA synthesis and many other metabolic processes (Rodwell et al., 2015). Both iron deficiency as well as excess of iron in the human body is problematic.

The most common nutritional deficiency world-wide is iron deficiency. The world's population affected by iron deficiency anemia was estimated to be 25% by the World Health Organization (McLean et al., 2009). Iron deficiency leads to fatigue, weakness, dizziness and circulatory collapse in extreme cases (Kasper et al., 2015). Poor dietary intake, infectious disease and chronic inflammation are the major causes of iron deficiency anemia (Wallace, 2016).

Free iron is highly reactive because it can readily accept or donate electrons. So, in the human body, the chemical reactivity of iron is directed and constrained by proteins and prosthetic groups association with iron (Ganz, 2013). Essentially, all circulating iron is bound to transferrin. This chelation renders the iron soluble and prevents iron-mediated free radical toxicity (Rodwell et al., 2015). So iron in excess can be toxic since it is a potent pro-oxidant. Hence, iron levels in the body need to be tightly regulated. Iron homeostasis is regulated

strictly at the level of intestinal absorption and release of iron from macrophages (Ganz, 2013)

Iron distribution in the body

The normal iron content of the body is about 3 to 4 g. Of this, about 2.5 g is contained in the hemoglobin in circulating red cells and in developing erythroblasts. Approximately, 400 mg of iron is in contained in iron-containing proteins such as myoglobin, cytochromes, catalase etc. About 3-7 mg of iron is bound to plasma transferrin. The rest of the iron in the body is stored as ferritin or hemosiderin.

Iron is stored commonly in the bone marrow, liver, and spleen. The primary physiologic source of reserve iron in the body is the iron stores of liver (Hentze et al., 2010a). Adult men have approximately 1 g of iron in storage. Adult women have less iron stores due to loss through menstruation, pregnancy and lactation. Only a small amount of iron enters and leaves the body on a daily basis. Most iron in circulation is recycled from the breakdown of old red blood cells by macrophages of the reticuloendothelial system (Camaschella and Schrier, 2017).

Iron absorption

Dietary iron exist as either heme or non-heme iron. Heme iron is obtained from foods of animal origin, such as red meats, fish, and poultry (Gropper and Smith, 2012). Humans are able to efficiently absorb heme (Tait, 2004). Iron in plant-based foods is not readily absorbable, as it is complexed in insoluble forms (non-heme iron) (Zimmermann and Hurrell, 2007). Daily absorption of iron is approximately 2 mg of iron. It occurs mainly in the duodenum and the proximal jejunum. Iron levels in the body are balanced by regulating the

absorption of iron, since there are no mechanisms to control the excretion of iron (Ganz, 2013; Rodwell et al., 2015).

Heme iron

Molecular mechanisms for intestinal absorption of heme are unclear. The proposed heme carrier protein 1, functions as a folate transporter (Qiu et al., 2006). This is highly expressed in the gut and stimulated by hypoxia (Shayeghi et al., 2005). A heme exporter, feline leukemia virus receptor 5 (FLVR5), is considered to export heme. FLVR5 is expressed in enterocytes, macrophages and erythroblasts (Keel et al., 2008).

Non-heme iron

Non-heme iron in the diet exists as ferric (Fe^{3+}) form; however, iron is absorbed only in ferrous (Fe^{2+}) form. The ferric form is reduced to its ferrous form by the reducing action of a membrane-bound ferric reductase, duodenal cytochrome B (Dcytb). This reductase is expressed on the apical brush border membrane of enterocytes (McKie et al., 2001). Divalent metal transporter 1 (DMT1) transports the ferrous iron across the apical membrane of intestinal epithelial cells. This is an integral transmembrane protein, which also helps in transportation of a number of other divalent cations apart from Fe^{2+} (Fleming et al., 1997; Gunshin et al., 1997).

Iron leaves the enterocyte and enters the systemic circulation with the help of the iron exporter, ferroportin. Iron is transported in circulation, in its ferric (Fe^{3+}) form, by the iron transport protein, transferrin (Rodwell et al., 2015). A membrane-bound protein, hephaestin, in the intestine oxidises Fe^{2+} to Fe^{3+}. This ferric iron immediately binds to apo- transferrin in the blood to become holo-transferrin (Vulpe et al., 1999). Cells take up iron from holo-transferrin and either use it for their requirements or store it as ferritin (Fe^{3+}) (Rodwell et al., 2015).

Iron transporter protein- transferrin

The gene for apotransferrin is situated on the long arm chromosome 3. Transferrin tightly binds one or two ferric (Fe3+) ions and is the major transporter of iron in plasma. It is mainly synthesised in the liver. The half-life of transferrin is 8 days. Its levels are increased in iron deficiency states; the underlying mechanisms for this are currently unknown (Beutler, 2010).

Circulating transferrin is approximately one-third saturated with iron under normal conditions (Cook, 1982; Finch and Huebers, 1982). Conditions with reduced transferrin saturation include iron deficiency anemia, anemia of chronic disease (anemia of inflammation) and patients with a ferroportin mutation (Kasper et al., 2015). Transferrin saturation is increased in hereditary and acquired hemochromatosis, aplastic anemia, conditions where the bone marrow is suppressed, , sideroblastic anemias, ineffective erythropoiesis, liver disease with reduced transferrin synthesis (Kasper et al., 2015) and monoclonal immunoglobulin with antitransferrin activity (rare) (Alyanakian et al., 2007).

Transferrin receptors

The transferrin (TfR) gene, which codes for a homodimeric transmembrane protein, is located on the long arm of chromosome 3. It is found in most cells, but most abundantly in erythroid precursors and placental cells (Rodwell et al., 2015).The first intron of the TfR gene harbours a binding site for the transcription factor Stat5 (Zhu et al., 2008). The TfR mRNA has five 3' IREs and is post-transcriptionally regulated by iron-regulatory proteins (IRPs). It is stabilized in conditions of iron deficiency and degraded in times of iron overload (Wang and Pantopoulos, 2011)..

Transferrin receptors are released from the surface of cells into the circulation via action of membrane proteases. These are called soluble TfR (sTfR). This is seen in cases of iron

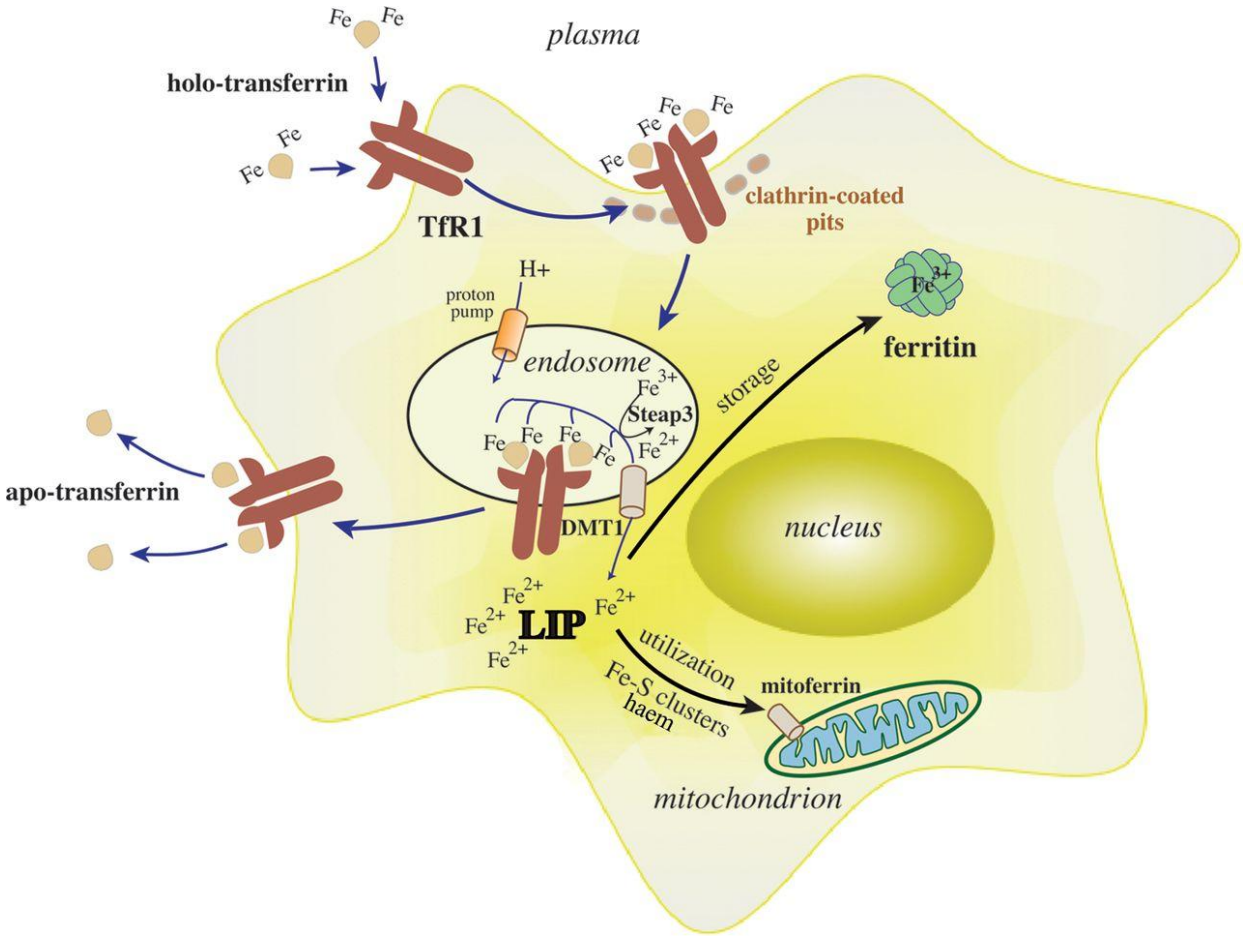
deficiency (Kaup et al., 2002). sTfR measures has been shown to correlate with erythropoietic expansion (Beguin et al., 1993).

Cellular iron uptake - the transferrin (Tf) cycle

Most cells, including developing erythroid cells, obtain iron from plasma transferrin (Tf). Iron-loaded holo-Tf binds, with increased affinity, to transferrin receptor 1 (TfR1) on the surface of cells (Ponka et al., 1998). It undergoes endocytosis via clathrin-coated pits. A proton pump boosts acidification of the endosome to pH 5.5, activating the release of Fe^{3+} from Tf that remains bound to TfR1. The ferrireductase, Steap3, reduces the ferric form to ferrous iron (Ohgami et al., 2005), which is transported across the endosomal membrane to the cytosol, by DMT1; in erythroid cells, it may also directly deliver iron to mitochondria (Richardson et al., 2010). After the release of iron from the endosome, the affinity of Tf for TfR1 decreases ~500-fold, resulting in its dissociation. In the last step of the cycle, apo-Tf is secreted back into the blood, ready to bind more ferric iron. The Tf cycle plays a vital role in delivery of iron to erythroid cells (Levy et al., 1999; Trenor et al., 2000).

Iron in excess of cellular requirements is stored in the form of ferritin. A cytosolic portion of intracellular iron that is redox-active constitutes the labile iron pool (LIP).

Figure 2.2. Cellular iron uptake via the Tf cycle



Wang, et al. 2010. Serum ferritin: Past, present and future. Biochim. Biophys. Acta 1800, 760–769.

TfR1- transferrin receptor 1

Two diferric Tf molecules (four Fe3+ atoms) bind to each TfR molecule

DMT1- Divalent metal transporter 1

LIP- labile iron pool

Ferroportin

Ferroportin (Ireg1, SLC40A1, formerly called SLC11A3, Mtp1) is a 12-transmembrane domain protein and is encoded by the gene *SLC40A1*(Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). It is the only known exporter of iron in mammalian cells (Donovan et al., 2005). It is found on the basal membranes of placental syncytiotrophoblasts, the basolateral surface of duodenal enterocytes, macrophages and hepatocytes (Abboud and Haile, 2000; Bastin et al., 2006; Donovan et al., 2005). It transports iron from the mother to fetus, allows macrophages to recycle iron from damaged and senescent red cells back into the circulation and transfers absorbed iron from enterocytes into the circulation. Its expression levels are regulated by both systemic and intracellular iron status. Systemic iron status is communicated through its interaction with hepcidin (Delaby et al., 2005; Donovan et al., 2005; Nemeth et al., 2004; Rivera et al., 2005). In animal and human in vitro models, the amount of available intracellular iron post-transcriptionally regulates ferroportin expression, due to the presence of a 5' UTR iron responsive element in the gene that encodes the protein (Chen et al., 2003; Delaby et al., 2005; Keel and Abkowitz, 2009; Zhang et al., 2009). In addition to this, both iron and erythrophagocytosis (through increase in heme content) have been shown to activate ferroportin transcription (Lakhal-Littleton et al., 2015).

Ferritin

Ferritin is a cellular storage protein for iron. It is a huge molecule with mol wt 440 kDa, with 24 subunit protein consisting of light (L ferritin, 20 kd, gene on chromosome 19) and heavy chains (H ferritin, 21 kd, gene on chromosome 11). It can store up to 4500 atoms of iron within its spherical cavity (Arosio and Levi, 2010). It has a ferroxidase activity that is needed for uptake of iron by the ferritin molecule. Iron is delivered to ferritin by an RNA binding

protein (poly (rC)-binding protein 1, PCBP1), which presumably works as a cytosolic chaperone (Shi et al., 2008). Apoferritin is the protein component of the molecule. Serum ferritin is usually apoferritin. Its plasma level represents iron stores. The presence of an iron overloaded state is indicated by an elevated serum ferritin level, in the absence of infection or inflammation (Kasper et al., 2015).

Ferritin, transferrin and the transferrin receptor are part of acute phase reactants. They play roles in cellular defence against oxidative stress and inflammation (Hintze and Theil, 2005; Wang et al., 2010).

Excretion of iron

Iron is lost from the body in sweat, desquamated cells from the skin and gastrointestinal tract. Losses amount to approximately 1 mg/day. Women sustain additional losses due to menstruation (Hentze et al., 2010a).

Iron homeostasis

Regulation of iron homeostasis occurs at systemic and cellular levels. Major cells involved in iron homeostasis of adults are duodenal enterocytes, macrophages and hepatocytes. Duodenal enterocytes absorb dietary iron. Macrophages recycle iron from erythrocytes and other cells. Hepatocytes store iron and release it when it is necessary (Ganz, 2013).

Systemic iron homeostasis

Systemic iron homeostasis is under the control of hepcidin, a peptide hormone secreted by the liver. Hepcidin is also known as liver-expressed antimicrobial peptide [LEAP-1] or hepcidin antimicrobial peptide [HAMP]). It is an acute phase reactant with intrinsic antimicrobial activity (Ganz, 2011; Nicolas et al., 2001; Pigeon et al., 2001; Stefanova et al.,

2017). It functions as a negative regulator of intestinal iron absorption and also iron release from macrophages(Rodwell et al., 2015). It binds to ferroportin, inducing internalization and lysosomal degradation of the molecule (Nemeth et al., 2004). Thus, hepcidin reduces iron absorption in the intestine and inhibits release of iron from macrophages (Ganz, 2011; Nemeth et al., 2004).

The breakdown of senescent red cells in the macrophages release approximately 20 to 25 mg of iron daily. Hemoglobin heme released from phagocytosed red cells, is catabolized by microsomal heme oxygenase to carbon monoxide and biliverdin. The iron is either released to the circulation through ferroportin or stored in ferritin according to the needs of the body and to the local concentration of hepcidin (Korolnek and Hamza, 2015). When released from ferroportin, ferrous iron is oxidized to the ferric form, and incorporated into transferrin. The oxidation process involves a copper-dependent multioxidase, ceruloplasmin (Rodwell et al., 2015).

Hepcidin is downregulated in conditions such as hypoxia, to allow increased iron through ferroportin. Hypoxia- inducible factor-2 (HIF-2α) increases the expression of several genes that encode for proteins involved in absorption of iron (Mastrogiannaki et al., 2013).

Intracellular iron homeostasis

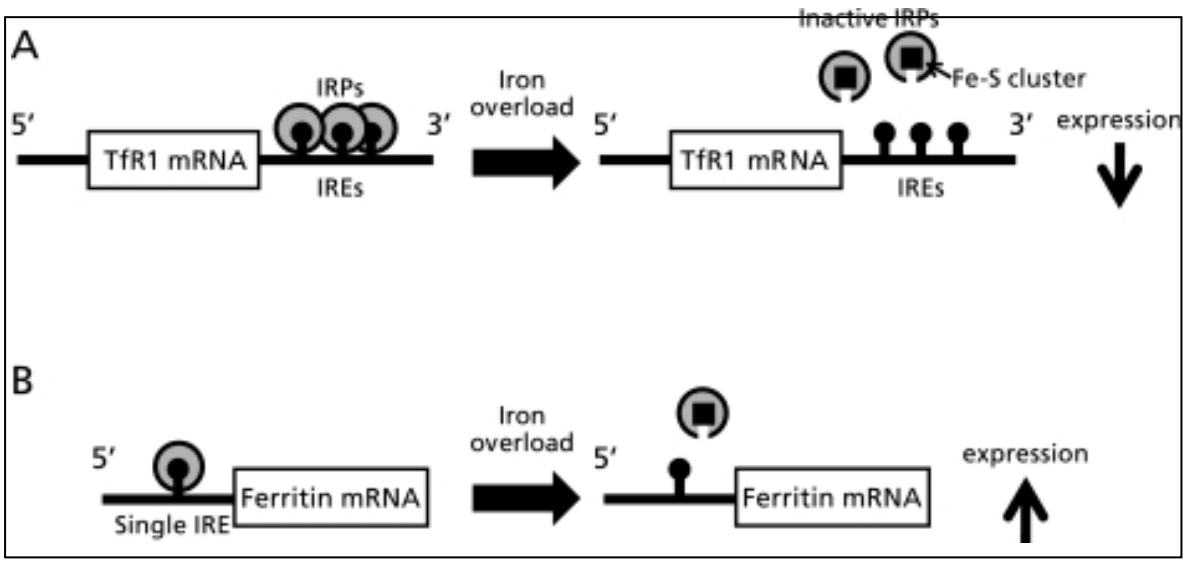
The iron status of a cell regulates the expression of proteins involved in cellular iron uptake and storage. Iron-regulatory proteins 1 and 2 (IRP1 and IRP2) are cytosolic RNA-binding proteins that attach to iron-responsive elements (IRE), which consisting of a loop configuration of nucleotides(Rodwell et al., 2015). IREs are found in the 5'- or 3'-untranslated regions (UTR) of specific mRNAs coding for genes that encode proteins involved in iron metabolism (eg, DMT1, ferritin, ferroportin, TfR, and the erythroid specific form of delta-

aminolevulinic acid synthase [eALAS]) (Hentze et al., 2010a). When a cell is iron-deficient, IRPs bind to IREs. It has varied effects depending on whether the IRE position is at the 3' or the 5' UTR, as described below (Hentze et al., 2010a). The rates of biosynthesis are decreased, when IRPs attach to the 5' IRE of ferritin, ferroportin, or eALAS. When IRPs attach to the 3' end of transcripts such as TfR or DMT1, the mRNA half-life is increased and rates of biosynthesis are increased.

When cellular iron content is increased, IRPs increase ferritin levels, so that excess iron can be stored; it also decreases expression of TfR1, in order to prevent further uptake of iron into the cell (Hentze et al., 2010a). Similarly, decreased intracellular iron results in low cellular ferritin and increased expression of TfR1 on the cell surface (Hentze et al., 2010a).

The state of the iron balance in the cell is detected by IRP1 and IRP2 in multiple ways. When the levels of cellular iron increase, assembly of iron-sulfur clusters occurs, with IRP1 acting as an aconitase; its ability to bind to IREs is lost. In the same state of increased levels of cellular iron, IRP2 interacts with a iron-stabilized protein, which recruits an E3 ligase (Salahudeen et al., 2009; Vashisht et al., 2009). This causes IRP2 to undergo ubiquitination and proteasomal degradation (Dix et al., 1992; Theil, 1993).

Figure 2.3. Intracellular iron homeostasis



Takami et al., 2011. Iron regulation by hepatocytes and free radicals. J. Clin. Biochem. Nutr. 48, 103–106.

IRPs -iron-regulatory proteins

IREs-insulin responsive element

TfR1- transferrin receptor 1

Serum iron parameters and T2DM

Increased serum ferritin levels have been reported to be associated with increased risk of T2DM (Fernández-Real et al., 2002a; Ford and Cogswell, 1999; Forouhi et al., 2007), gestational diabetes (Afkhami-Ardekani and Rashidi, 2009), pre-diabetes (Sharifi et al.,

2008), metabolic syndrome (MetS)(Jehn et al., 2004), central adiposity (Gillum, 2001), and cardiovascular disease (Iwasaki et al., 2005; Qi et al., 2007). It has also been observed that phlebotomy improves glycemic control in patients with diabetes mellitus, hemochromatosis and MetS (Bofill et al., 1994; Fernández-Real et al., 2002b).

Iron-related proteins in adipose tissue

Ferroportin knock-down in adipose tissue has been shown to result in increased iron content in the adipose tissue, insulin resistance and fasting hyperglycemia (Gabrielsen et al., 2012). On the other hand, it has also been observed that iron deficiency anemia is prevalent in obese patients with T2DM (Fernández-Real et al., 2015). However, very little is known about the role of iron metabolism in adipose tissue in the development of insulin resistance.

A study done by Moreno-Navarrete et al (2014) showed that in a cohort of morbidly obese patients undergoing bariatric surgery, ferritin light chain (FTL) mRNA and protein levels and ferroportin transcripts were significantly increased, whereas transferrin mRNA decreased, suggesting increased iron levels in adipose tissue. In the same study, it was shown that following bariatric surgery–induced weight loss, transferrin mRNA was increased and FTL and ferroportin were decreased in subcutaneous adipose tissue, and this was associated with improved insulin sensitivity (J. M. Moreno-Navarrete et al., 2014).

Pihan-Le Bars et al (2016) have found that the iron content in subcutaneous and visceral adipose tissue of obese patients appears to be increased and it was negatively correlating with adiponectin expression. So they have concluded that this could contribute to insulin resistance which is a metabolic complication of obesity (Pihan-Le Bars et al., 2016).

THE STUDY

Background

Type 2 diabetes mellitus (T2DM) has been shown to be associated with increased body iron stores. Iron levels in adipose tissue have been postulated to play a role in the pathogenesis of insulin resistance.

Hypothesis

Iron metabolism in adipose tissue from patients with diabetes mellitus may be different from that in adipose tissue from those without diabetes mellitus.

Aim

The aim of the study was to test the hypothesis that patients with T2DM show alterations in iron metabolism in their adipose tissue and in iron-related parameters in blood compared to those without T2DM.

Objectives of the study

1. To determine mRNA expression of transferrin receptor 1(TfR1) (the iron import protein) and ferroportin (the iron export protein) in subcutaneous and visceral adipose tissue in patients with T2DM and in control subjects
2. To compare serum levels of iron, ferritin and transferrin saturation in patients with T2DM and control subjects
3. To obtain anthropometric data of these patients and correlate these with the above parameters

MATERIALS

Equipment used

1. Elix and Milli-Q ultrapure water systems (Millipore, USA)
2. Table-top refrigerated centrifuge (MPW R 350, MPW Poland)
3. -70^{0}C freezer (Thermoscientific, Massachusetts, USA)
4. Glass homogenizer with Teflon pestle (1 mL capacity) (Kimble-Kontes, USA)
5. Agarose gel electrophoresis system (Medox Biotech, India)
6. Gel documentation system (Alpha Innotech, USA)
7. Applied Biosystem 2720 Thermocycler (ThermoFisher Scientific) for cDNA construction
8. Real-time thermo cycler (Chromo4, Bio-Rad, USA) for qPCR
9. Nano-drop spectrophotometer (Thermo Scientific, USA)

Chemicals and reagents used

1. TRI Reagent, diethyl pyrocarbonate (DEPC), ethidium bromide, ethylene diamine tetraacetic acid (EDTA), formamide, formaldehyde, bromophenol blue and sodium hydroxide were obtained from Sigma, India.
2. Absolute alcohol was obtained from Hayman Ltd, England.
3. Agarose was obtained from Genei, Bangalore, India.
4. 3–morpholinopropane sulfonic acid (MOPS) was purchased from Sigma, India.
5. Reverse transcription core kit, SYBR Green PCR master mix kit was obtained from TaKaRa Bio, USA

6. Gene specific primer for beta-actin was obtained from Sigma, India and gene specific markers for ferroportin and TfR1 from Eurogentec, Belgium.

All chemicals used were of analytical grade**.**

Miscellaneous consumables used

1. Vacutainer tubes for blood collection (BD Biosciences, Plymouth, UK).
2. Micro-tubes and centrifuge tubes (1.5mL, 0.5 mL) (Tarsons Products Private Limited, Kolkata, India).
3. Micro tips (Tarsons Products Private Limited, Kolkata, India).

METHODS

This study was approved by Institutional Review Board (IRB) of Christian Medical College, Vellore (IRB minute number- 9902, dated February 5th 2016). The letter of approval is shown as Appendix I.

Study design:

Case control study

Setting

Department of Biochemistry, Surgery Unit 3 and Surgery Unit 4 of Christian Medical College, Vellore.

Period of sample collection was from 12th July 2016 to 6th July 2017.

Participants

Eligibility criteria

Patients, who underwent elective abdominal surgery in Surgery Unit 3 and Unit 4 of Christian Medical College, Vellore, if they met the following criteria:

Inclusion criteria

Patients, between the ages of 19-61 years and of both genders

Willing to give informed consent to participate in the study

Exclusion criteria:

Patients with documented evidence of complications of diabetes, such as retinopathy, nephropathy or neuropathy.

Patients with Hb < 10gm/dL (Bohlius et al., 2006).

Those who have declined to give informed consent.

History or clinical evidence of chronic liver or kidney disease, chronic inflammatory diseases or any malignancy.

Informed consent

Patients, who satisfied inclusion and exclusion criteria as listed above, were invited to participate in the study. The study was explained to each patient. They were also provided with an information sheet in their own language or in English, according to their preference. After that, written consent was obtained from each patient who expressed their willingness to participate (informed consent form enclosed in Appendix II).

They were categorized into cases and controls as indicated below, using criteria of the American Diabetic Association (ADA) (2015) for diagnosis of diabetes mellitus among the cases.

Cases	Controls
Recruited patients who had been diagnosed to have Type 2 DM (and on treatment or with an HbA1c level of > 6.5%)	Recruited patients who were found to be non-diabetic (no history of type 2 diabetes mellitus or with a HbA1c level of < 5.7%)

Calculation of sample size

There are very few published studies that have studied the expression of iron-related proteins in adipose tissue. Pihan-Le Bars et al. (2015) have studied markers of iron status in adipose tissue of patients who were morbidly obese. They studied 16 obese subjects and 30 control subjects. There is no information in this publication on mean values and standard deviations of the parameters of interest, to enable calculation of a sample size based on this data. There are no other publications we have been able to access to use as a basis for a sample size calculation. Hence, we set an arbitrary goal to recruit 20 diabetics and 20 non-diabetics for this study.

Patient data

A proforma was prepared to collect relevant patient data. This is included in Appendix III.

Clinical and laboratory data

The investigator elicited relevant history from each patient. Clinical data, including results of relevant blood tests for patients, were obtained from their hospital records.

Anthropometric data

The weight of each subject was measured to the nearest 0.5 kg, with the use of a weighing scale available in the surgery ward. The height of each subject was measured, to the nearest centimeter, with the use of a tape stuck to the wall with the head positioned in the Frankfurt plane. The body mass index (BMI) of each patient was calculated, using the following formula:

$$\text{BMI} = \text{body weight (in kg)} \div \text{height (in meters)}^2$$

Sample collection

Blood sample

Blood samples were collected from patients by venipuncture on the day of the surgery, with the co-operation of the anaesthetists and surgeons concerned. Blood was collected in BD vacutainer tubes. Approximately 6 ml of blood was collected from each patient. The blood sample collected was used for estimation of serum levels of iron and ferritin, total iron-binding capacity and transferrin saturation. An additional 2ml collected from all the patients who did not have a history of T2DM, in order to measure HbA1c levels. Parameters such as hemoglobin (Hb) and serum creatinine levels were obtained from the hospital records of the patients.

Samples of adipose tissue

At the time of surgery, samples of sub-cutaneous adipose tissue (from the anterior abdominal wall) and visceral adipose tissue (from the omentum) were collected, under aseptic conditions. Part of each tissue sample obtained was snap-frozen in liquid nitrogen and another put into TRI Reagent and placed on ice. They were transported to the research laboratory in the Department of Biochemistry and stored at minus 70^{o}C till analyses. These samples were used for the determination of gene expression of ferroportin and TfR1, by quantitative PCR.

Processing and storage of blood samples

To obtain serum, the clotted blood was centrifuged at 2500 rpm, within 2 hours of blood collection. Serum obtained was divided into multiple aliquots and stored at -70^{o}C. When required, samples were thawed to room temperature and used for estimation of serum iron,

UIBC and ferritin. EDTA tubes were taken immediately to the Department of Clinical Biochemistry, CMC, Vellore, for the measurement of HbA1c.

Estimation of HbA1c

***Analyzer used*:** BIO-RAD VARIANT II TURBO

***Principle of method*:**

Estimation of HbA1c was done using BIO-RAD VARIANT II TURBO HbA1c Kit – 2.0. This kit was based on the ion-exchange high performance liquid chromatography (HPLC). Whole blood EDTA samples were automatically diluted in the sampling station and injected into the analytical cartridge. A buffer of increasing ionic strength was pumped into the cartridge and helped in separation of hemoglobin, based on their ionic interactions. The change in absorbance of the separated hemoglobin flowed through the filter photometer was measured at 415nm and 690nm (to correct background disturbance).

The VARIANT II TURBO clinical data management software generated a chromatogram and a report of retention times of detected peaks for each sample. Exponentially modified Gaussian (EMG) algorithm was used for the calculation of A1c peak to exclude labile A1c and carbamylated peak area.

Reference interval

Normal range: HbA1c < 5.7%

Prediabetics: HbA1c between 5.7% and 6.4%

Diabetic: HbA1c ≥ 6.5 %

Estimation of serum ferritin

Analyzer used: Siemens, ADVIA Centaur Immunoassay system Xpi, UK

Principle of the method: Two-site sandwich immunoassay using direct chemiluminescence technology

Two anti-ferritin antibodies were used in this method. The first antibody was polyclonal goat anti-ferritin antibody, labeled with acridinium ester. Monoclonal mouse anti-ferritin antibody, covalently coupled to paramagnetic particles was the second antibody. These antibodies were sequentially added to the reaction chamber. These antibodies bound the ferritin molecule present in the serum sample. On adding substrate (0.1 N nitric acid, 0.5% hydrogen peroxide and alkaline medium), acridinium ester was excited and released a photon, which was measured in terms of relative light units (RLU).

The amount of ferritin present in the sample was directly proportional to the amount of RLUs detected by the system.

Reference interval

Men and women > 50 years: 20-320 ng/mL

Women < 50 years: 10-290 ng/mL

Estimation of unsaturated iron binding capacity (UIBC)

Analyzer used: Roche Cobas 8000c 702 modular analyzer

Principle of the method

A known amount of ferrous iron was added to the sample at an alkaline pH. The ferrous ions bound to transferrin at unsaturated iron binding sites. The unbound ferrous ions were measured using the ferrozine method (described above under the estimation of serum iron).

The difference between the amount of ferrous ions added to the sample and the unbound ions measured was taken to be the unsaturated iron binding capacity (UIBC) of the sample.

UIBC = [Amount of ferrous ion added] - [Amount of unbound ferrous ion]

TIBC was calculated as the sum of serum iron concentration and the UIBC

TIBC = Serum iron + UIBC

Reference interval

Males - 300-400 µg/dL

Females - 250-350 µg/dL

Estimation of serum iron

Analyzer used: Roche Cobas c 702 modular analyzer

***Principle of the method*:** Guanidine/ ferrozine spectrophotometric method

Transferrin-bound ferric ions in the sample were released by guanidine, and reduced to ferrous form by means of hydroxylamine. Ferrous ions reacted with ferrozine to form a purple-colored complex. The absorbance of the sample was measured at 560 nm, using spectrophotometry. The intensity of the color obtained was directly proportional to the concentration of iron in the sample.

Reference interval

Male - 60- 160 µg/dL

Female - 40-145 µg/dL

Calculation of transferrin saturation (TSAT)

It was calculated as the ratio of serum iron and total iron binding capacity, multiplied by 100.

TSAT = (Serum iron / TIBC) ×100

Processing of tissue samples

Isolation of RNA from the tissue samples

The samples of adipose tissue (both subcutaneous and visceral) collected in TRI Reagent and which were stored at -70^{o}C were used for isolation of RNA. This was done according to the manufacturer's instructions.

1. The tissue was homogenised in the TRI-Reagent.
2. The homogenised sample was subjected to centrifugation at 2,000*g* for 10 minutes, at 4^{o}C.
3. The supernatant was taken and it was transferred to new tube for further processing
4. Chloroform (160 µl for 800 µL of TRI-Reagent) was added to each tube and mixed for 15 seconds.
5. The mixture was kept at room temperature for 15 minutes.
6. It was then centrifuged at 12000*g* for 20 minutes, at 4^{o}C. Centrifugation separated the mixture into 3 phases; the upper aqueous phase contained RNA.
7. The aqueous phase was transferred to a fresh microtube, and isopropanol (400µL for 800 µL of TRI-Reagent) was added to it and mixed.
8. The mixture was kept at -20^{o}C for 15 minutes.
9. It was then centrifuged at 12000*g* for 10 minutes, at 4^{o}C. RNA that was precipitated formed a pellet on the side and bottom of the tube.

10. The supernatant was discarded and the RNA pellet was washed by adding ice cold 70% ethanol 800 µl for 800 µl of TRI-Reagent
11. This was centrifuged at 7000*g* for 5 minutes at 4^{o}C.
12. The supernatant was discarded; the RNA pellet obtained was air-dried for 5-10 minutes, by placing them on ice and keeping the caps of the tubes open.
13. DEPC water (20µL) was added to each tube with the RNA pellet; the sample was kept at 60^{o}C for an extra 10 minutes if the pellet was not dissolved.
14. The concentration of RNA in each sample was quantitated, using a nano-spectrophotometer.

RNA quantitation

A nano-spectrophotometer was used to estimate the quantity of RNA in the samples.

Principle: Nucleic acids absorb ultraviolet light strongly at a wavelength of 260nm. An optical density reading of 1.0 at 260 nm indicates an RNA concentration of 40µg/mL.

DNA and protein contamination of the isolated RNA was confirmed. The ratio of absorbance at 260 and 280 nm and at 260 and 230 nm was used to assess the purity of RNA. If the A260/280 ratio was less than 1.8, or if there was evidence of phenol or guanidium isothiocyante contamination, the RNA was repurified by precipitation using ethanol.

Ethanol precipitation

1. An aliquot (20 µL) of the RNA obtained was diluted to 100µL by adding 80 µL DEPC water.
2. Sodium acetate (3M) 10µL (0.1 part by volume) was added and mixed using a vortex mixer.

3. Ice-cold ethanol (100%) (220 µL -2.2 parts by volume) was added and mixed thoroughly.
4. The tubes were kept at -20^{o}C overnight
5. After overnight freezing, the tubes were centrifuged at 12000 g for 10 minutes
6. Ice cold ethanol (70%) (500 µL) was added to each tube.
7. The tubes were centrifuged for 5 minutes at 12000 g
8. The supernatant was discarded; the RNA pellet obtained was air-dried for 5-10 minutes, by placing them on ice and keeping the caps of the tubes open.
9. DEPC water (20µL) was added to each tube with the RNA pellet; the sample was kept at 60^{o}C for an extra 10 minutes if the pellet was not dissolved. The concentration of RNA in each sample was quantitated, using a nano-spectrophotometer.

Once the samples were confirmed to be free of DNA and protein contamination, the integrity of the RNA was assessed. 10 samples which showed evidence of contaminations even after ethanol precipitation were excluded from the study.

The integrity of the RNA isolated was confirmed by gel electrophoresis.

Assessment of RNA integrity by gel electrophoresis:

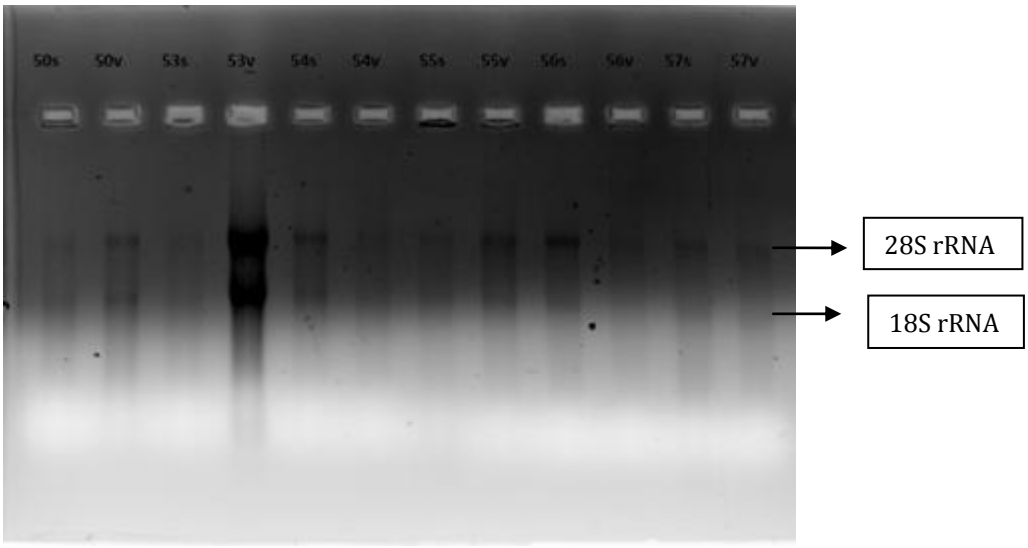
The integrity of the isolated RNA was confirmed by agarose gel electrophoresis.

1. 10X MOPS (morpholino-propanesulfonic acid) was prepared.
   In order to prepare 100 mL, 4.186 g of MOPS was dissolved in DEPC water. The pH of the solution was adjusted to 7.0, using 0.1 M NaOH.
   Sodium acetate (0.6804 gm) was then added to it (to give a final concentration of 50mM) and 2mL of 0.5M EDTA (to obtain a final concentration of 10nM).
2. Preparation of agarose gel for electrophoresis (40mL of 1.2% gel)

a. To 34 mL of DEPC-treated water in a conical flask, 0.48 g of agarose was added. This was heated till the mixture boiled and the agarose melted.
b. To the melted agarose, 2.15mL of formaldehyde, 4 mL of 10 X MOPS, and 7µL ethidium bromide were added and mixed.
c. This mixture was poured into a gel casting tray, combs were inserted into the mixture and it was allowed to set for 1 hour.

3. Running buffer was prepared by 30 mL of 10X MOPS and 270 mL of DEPC-treated water
4. Preparation of the sample
    a. 10µl of each RNA sample was mixed with 2.5µL of 10X MOPS, 3.5µl of formaldehyde and 10µL of formamide.
    b. The sample mixture was incubated at 60^0C in a dry bath for 15 minutes.
    c. After incubation at 60^0C for 15 minutes, 3µl of bromophenol blue was added to the sample mixture.
5. The cast gel was placed in the buffer in the electrophoresis tank; samples were loaded into the wells in the gel.
6. Samples were kept for electrophoresis at 150 volts for 45 minutes.
7. The RNA bands separated were visualized using an ultraviolet transilluminator in a Protein Simple gel documentation system. Two distinct bands were seen, which represented the 28S and 18S ribosomal subunits of RNA. When the 2 bands in each sample were found in an approximate band density ratio of 2:1, this was considered as evidence of RNA of good quality.

Representative images of RNA bands obtained on agarose gel electrophoresis



cDNA construction by reverse transcription

Reverse transcription of RNA was done using Prime script 1st strand cDNA synthesis kit TaKaRa Bio, USA

Principle

In the presence of dNTP, random nanomers and reaction buffer, the reverse transcriptase enzyme converts RNA into cDNA.

Components of the kit

For 200 reactions

i. 5X PrimeScript Buffer 400 µL (contains dNTP Mixture and Mg^{2+})
ii. PrimeScript RT Enzyme mix I 100 µL
iii. Oligo dT Primer (50 µM) 100 µL
iv. Random hexamers (100 µM) 400 µL
v. RNase free H2O 1 mL

Steps

1. The following reaction mixture was prepared and placed on ice

   For 1 reaction

	Reagent	Amount
i.	5x Prime script buffer	2µL
ii.	RT enzyme mix	0.5µL
iii.	Oligo dT primer	0.5 µL
iv.	Random 6mers	0.5 µL

   A master-mix was prepared by adding together all the above reagents in the proportions mentioned, for the required number of samples
2. A volume of sample containing 500 ng RNA was added to a microtube.
3. DEPC water was added to each microtube so that the volume of RNA + DEPC water was 6.5 µL. Master Mix (3.5 µL) was added to it to make the final volume of 10 µL.
   a. RNA+DEPC water= 6.5 µL
   b. Master Mix =3.5 µL
4. Preparation of the negative controls Negative controls were also set up which were as follows:
   a. No template: This tube contained all the reagents except the RNA template. This was to confirm that the reagents and consumables used were not contaminated with DNA.
   b. No reverse transcriptase: This was a tube that contained all the above reagents except the reverse transcriptase. This negative control was used to confirm that there was no DNA contamination of the RNA sample used.
5. All the reaction tubes were mixed and centrifuged in a microfuge for a few minutes.
6. The tubes were incubated under the following conditions:

For reverse transcription	37^0C for 15 minutes
Inactivation of reverse transcriptase	85^0C for 5seconds
Cooling of samples	4^0 C for 10 minutes

The cDNA obtained were stored at -20^0C till real-time polymerase chain reaction analysis

Real-Time Polymerase Chain Reactions (PCR) or quantitative PCR (qPCR)

The cDNA obtained from subcutaneous and visceral adipose tissue samples were amplified by real time PCR assays, for ferroportin and TfR1 genes. Beta-actin was used as the house-keeping gene. The PCR reactions were set up in 96 well plates. To ensure reproducibility all samples were assayed in quadruplicates.

Gene-specific primers

The following gene-specific primers were used for the reactions.

Gene		Primer sequence	Reference
Beta-actin	Forward	5'-AGAGCTACGAGCTGCCTGAC -3'	(Wang et al., 2013)
	Reverse	5'-AGCACTGTGTTGGCGTACAG -3'	
Ferroportin	Forward	5'- TGACCAGGGCGGGAGA -3'	(Theurl et al., 2006)
	Reverse	5'- GAGGTCAGGTAGTCGGCCAA-3'	
TfR1	Forward	5'- TCCCAGCAGTTTCTTTCTGTTTT-3'	(Theurl et al., 2006)
	Reverse	5'-CTCAATCAGTTCCTTATAGGTGTCCA-3'	

Primer Blast

Primer Blast software was used to check specificity of the forward and reverse primers of the genes of interest. Information regarding annealing temperature, amplicon length and length of the primers was also obtained from this source.

Gene	**Length of the primers**	**Amplicon length**
Beta-actin	Forward primer:20 Reverse primer:20	**184**
TfR1	Forward primer: 23 Reverse primer:26	**86**
Ferroportin	Forward primer:16 Reverse primer:20	**67**

Standardization of PCR conditions

PCR conditions were standardized using the relative standard curve method. In order to do this, a standard curve for each gene of interest was constructed for SAT and VAT samples separately. Pooled cDNA, obtained from 20 randomly selected SAT / VAT samples (1 μL from each sample), was used for this purpose. Serial dilutions were made as shown in the following table.

S1	20 µL of pooled cDNA	Undiluted
S2	4 µL S1+16 µL of DEPC water	1:5
S3	4 µL S2+16 µL of DEPC water	1:25
S4	4 µL S3+16 µL of DEPC water	1:125
S5	4 µL S4+16 µL of DEPC water	1:625
S0	20 µL of DEPC water alone	Blank

PCR reactions were run for S1 to S6 (in duplicates) and the results obtained were analyzed.

qPCR validation data for SAT

Sl. No	Gene	Standard curve slope	R^2 of standard curve	Linear dynamic range (cDNA dilution)	Primer dimer (melting curve analysis)	Ct of amplification (if any) in the NTC*
1	Beta actin	-3.405	0.997	1: 5 to 1: 625	in NTC	37
2	Ferroportin (Slc40a1)	-3.065	0.997	1 to 1: 625	No	None
3	TfR1 (Transferrin receptor 1)	-3.08	0.994	1 to 1:625	No	None

* NTC – no template control

qPCR validation data for VAT

Sl. No	Gene	Standard curve slope	R^2 of standard curve	Linear dynamic range (cDNA dilution)	Primer dimer (melting curve analysis)	Ct of amplification (if any) in the NTC*
1	Beta actin	-3.362	1	1 to 1:625	No	None
2	Ferroportin (Slc40a1)	-3.083	1	1 to 1: 625	No	None
3	TfR1 (Transferrin receptor 1)	-3.238	0.999	1:5 to 1: 625	No	None

* NTC – no template control

For both SAT and VAT, primer pairs for all the 3 genes analyzed (beta actin, TfR1 and ferroportin) showed good amplification efficiency (as indicated by the slope of the standard curve which was within the acceptable range [-3.32 ± 0.3]). In all cases, amplification was linear (as indicated by $R^2 > 0.995$) with a dynamic range of up to 1: 625 dilution of cDNA.

Dilution of cDNA

cDNA (5 µL) was diluted with 45 µL of DEPC water (1:10 dilution) and used for PCR reactions.

Steps in qPCR

1. Preparation of the master mix

   Components of the master mix to perform a single PCR reaction were as follows.

Component	**Volume**
Diluted cDNA template	2μL
SYBR green master mix	5 μL
Gene specific primer mix	0.25 μL
DEPC water	2.75 μL

   A master mix was prepared with the above components in specified proportions for 106 reactions (96 reactions + 10% extra for pipetting loss).

2. 8 μL of the master mixture was added into each of 96 wells followed by addition of 2 μL cDNA.

Each sample of cDNA was assessed for 3 genes namely beta-actin, ferroportin, and TfR1. To ensure reproducibility all samples were assayed in quadruplicates. PCR assays using cDNA obtained from visceral and subcutaneous tissue were done separately.

3. After adding the cDNA and master mix, the wells in the plate were closed tightly, using transparent caps.
4. The contents of each well were mixed, and the plate was briefly centrifuged for 2 minutes at 2000 RPM. The plate was then placed in the thermal cycler, which was programmed as shown below.

Thermal cycler program

Step		**Temperature**	**Time**
1	Hot start	95^0 C	3 minutes
2	Denaturation	95^0 C	10 seconds
3	Annealing and extension	60^0 C	60 seconds
4	Reading taken		
5	Steps 3, 4 and 5 were repeated for 39 more cycles		
6	From 50-95^0 C, melting curve analysis was done. Readings were taken every 10 seconds for every 1^0 C increase in temperature.		
7	Within the thermocycler, samples were cooled and maintained at 4^0 C for 10 minutes		

After completion of PCR assays for each gene of interest, the log fluorescence data graph and melting curves were obtained.

Calculation of levels of gene expression

Samples were run in quadruplicates and the mean Ct was calculated for each sample using the MJ Opticon Monitor PCR analysis software (BioRad, USA). Gene expression was calculated by the $2^{-\Delta\Delta}$Ct method (relative Ct method) as described below.

Calculation of ΔCt value

Expression levels of the genes of interest (TfR1 and ferroportin) were normalized to that of beta actin, which was used as the internal reference gene. Normalization was done by subtracting the Ct value for beta actin obtained for each sample from the Ct values obtained for the genes of interest in the same sample (ΔCt value).

Calculation of ΔΔCt value

For each gene of interest, the average ΔCt of control samples was calculated. This value was subtracted from ΔCt of each sample (both controls and cases) in order to obtain the ΔΔCt.

Calculation of fold-change

Fold-change in gene expression was calculated using the following formula:

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

The MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) check-list, a set of guidelines that describe the minimum information necessary for reporting qPCR experiments, is provided as Appendix IV.

Statistical methods

The Statistical Package for the Social Sciences (SPSS), version 16, was used for the analysis of data obtained. In order to determine the distribution of the data, the Shapiro Wilk's test was used. Data that were normally distributed were expressed as mean and standard deviation. Data with a skewed distribution were expressed as medians and interquartile ranges. The independent t-test was used for the comparison of normally distributed data, while the Mann-Whitney U test was used for skewed data. Bivariate correlation analyses were done using Pearson correlation for variables with normal distribution and Spearman's rank correlation for variables that are not normally distributed. A P value of less than 0.05 was taken to indicate statistical significance in all cases.

RESULTS

Distribution of data

Data on age, Hb, serum creatinine, HbA1c, serum iron, TIBC, transferrin saturation were found to be normally distributed. Data on BMI, serum ferritin, TfR1 and ferroportin mRNA expression in SAT, and TfR1 and ferroportin qPCR in VAT were found to have skewed distributions. Data with normal distribution was expressed as mean ±SD, and the unpaired t test was used for comparison of data in the 2 groups. Skewed data was expressed as median and inter-quartile ranges (IQR) and the Mann-Whitney U test was used for comparison of data in the 2 groups. A p value of < 0.05 was taken to indicate statistical significance in all cases.

Characteristics of subjects of the study

A total of 37 subjects were included in the study. Twenty three patients were diabetic, while fourteen were control subjects. The characteristics of the subjects are shown in Table 1.

Table 6.1. Characteristics of subjects in the study

Characteristics	**Control subjects (n=14)**	**Diabetic subjects (n=23)**	**P value**
Age (years)	42 ± 7.5	51 ± 6.0	0.001
Sex (M/F)	5/9	8/15	
BMI (kg/m^2)	29.6 (22.79 - 34.1)	27.6 (25.9 - 32.8)	0.661

Data shown as mean ± SD or as median and inter-quartile ranges (IQR)

Among the control subjects, five were males and nine were females. Among the diabetics, eight were males and fifteen were females. The diabetic subjects were significantly older than the control subjects. The mean BMI were similar in the 2 groups.

Table 6.2. Results of relevant laboratory parameters of subjects in the study

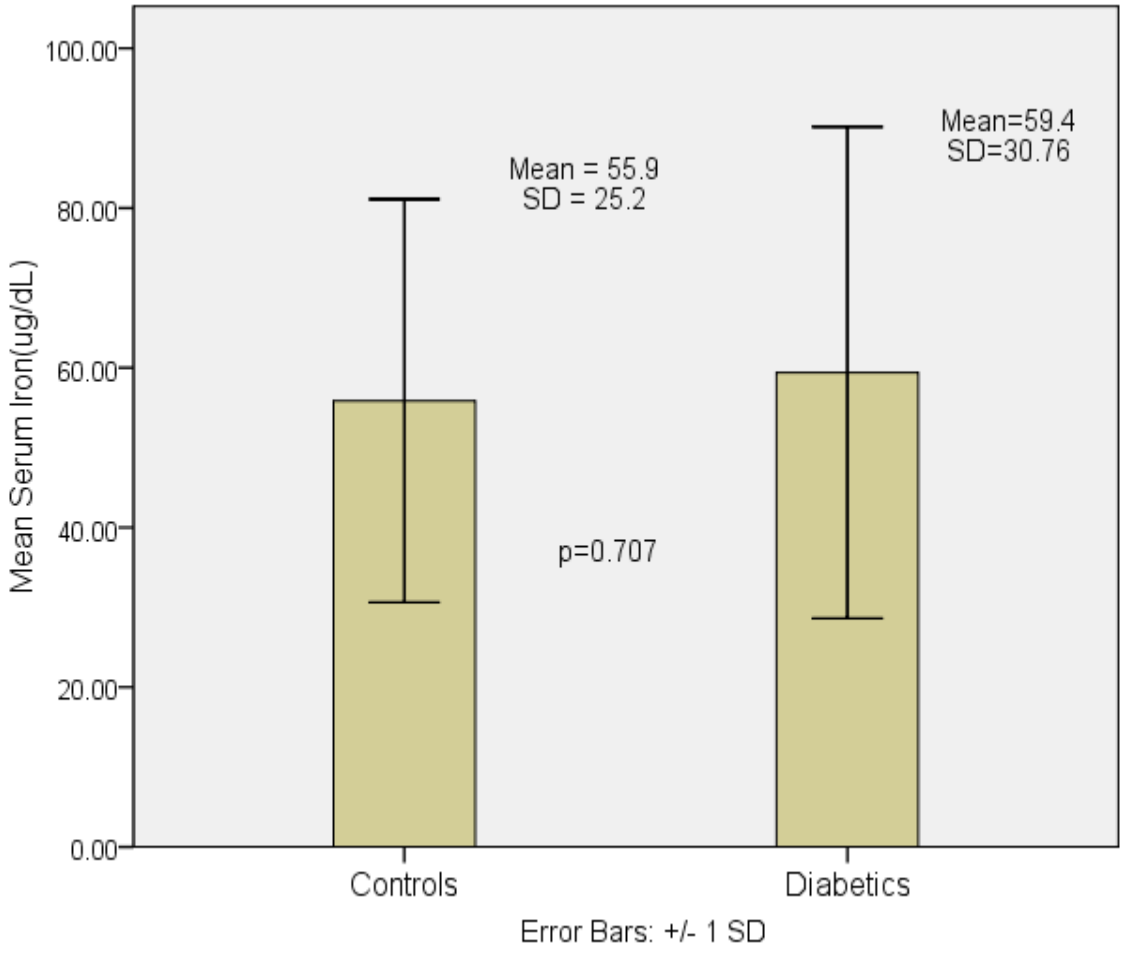
Characteristics	**Control (n=14)**	**Diabetic (n=23)**	**P value**
Hb (g/dL)	12.5 ± 1.04	12.8 ± 1.56	0.491
HbA1c (%)	5.3 ± 0.3	8.5 ± 1.6	<0.001
Serum creatinine (mg/dL)	0.75 ± 0.11	0.66 ± 0.17	0.037

Data shown as mean ± SD

Hemoglobin levels were similar in diabetics and control subjects. The level of glycated hemoglobin (HbA1c) was significantly higher in the diabetic group than in the control subjects, while serum creatinine was significantly higher in the control group.

The results of serum parameters of iron status (serum iron and ferritin, TIBC and transferrin saturation) are shown in the figures below.

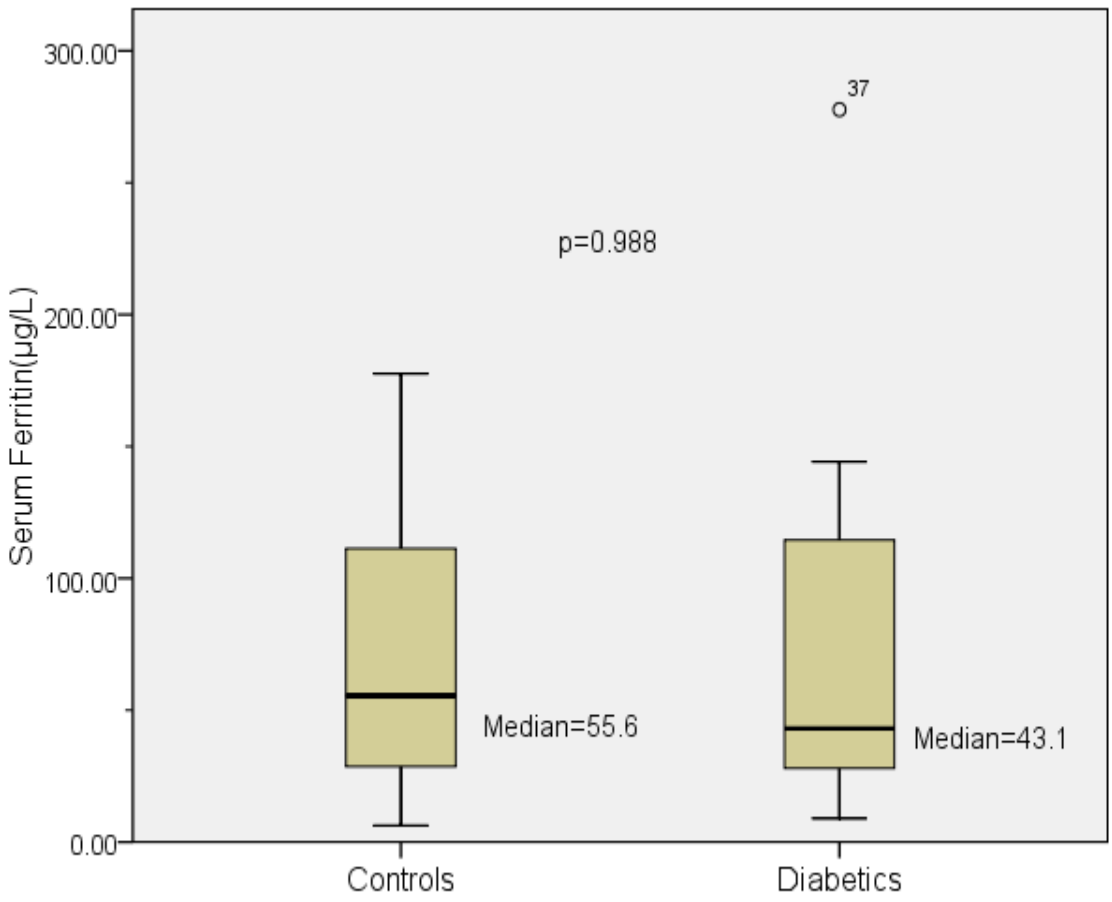
Figure 6.1. Serum iron levels in control subjects and diabetics



Data are expressed as mean ±SD.

Serum iron levels were similar in the control and diabetic subjects.

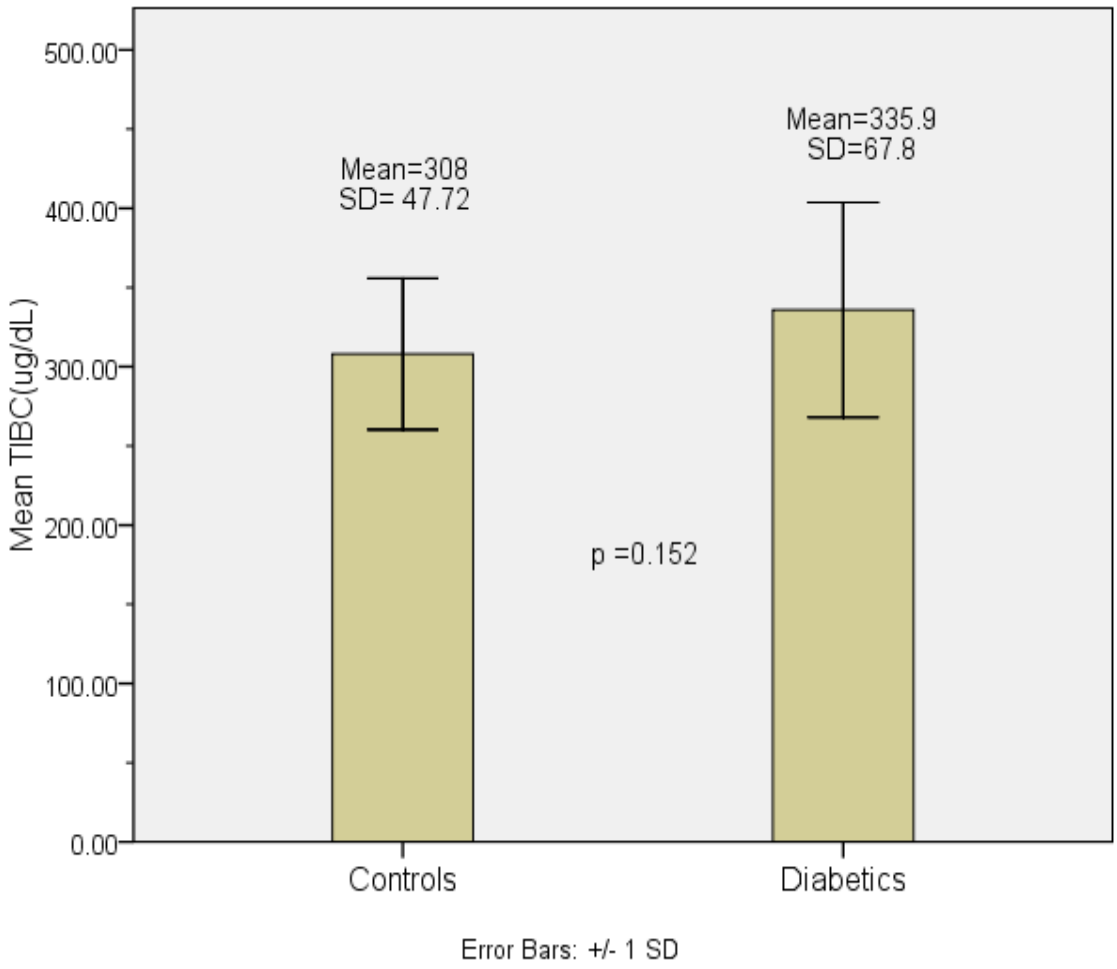
Figure 6.2. Serum ferritin levels in the control and diabetic groups



Data are shown as box and whisker plots, with medians and quartiles shown.

Serum ferritin levels were similar in the 2 groups.

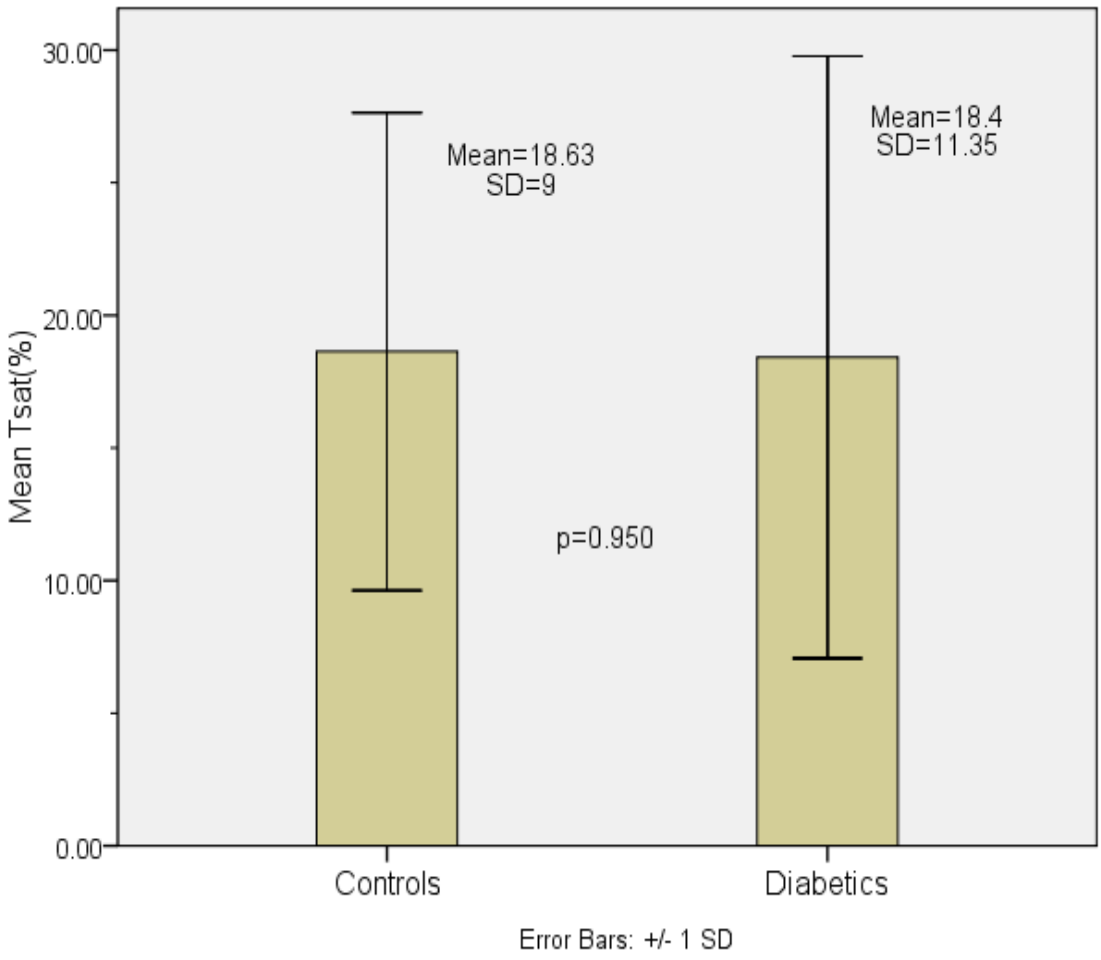
Figure 6.3. TIBC values in the control and diabetic groups



Data is expressed as mean ±SD

TIBC levels were similar in the two groups.

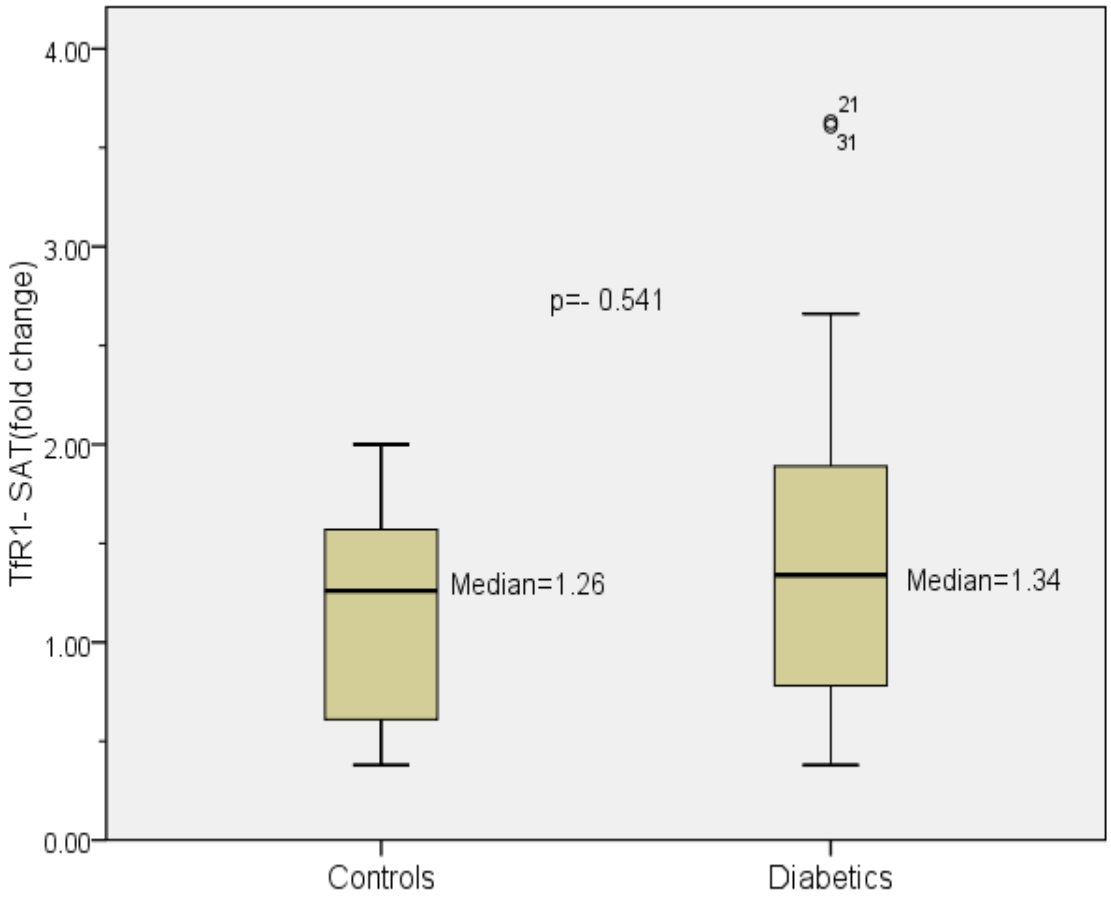
Figure 6.4. Transferrin saturation levels in the control and diabetic groups



Data are expressed as mean ±SD

The mean transferrin saturation (T sat) levels were found to be similar in the 2 groups.

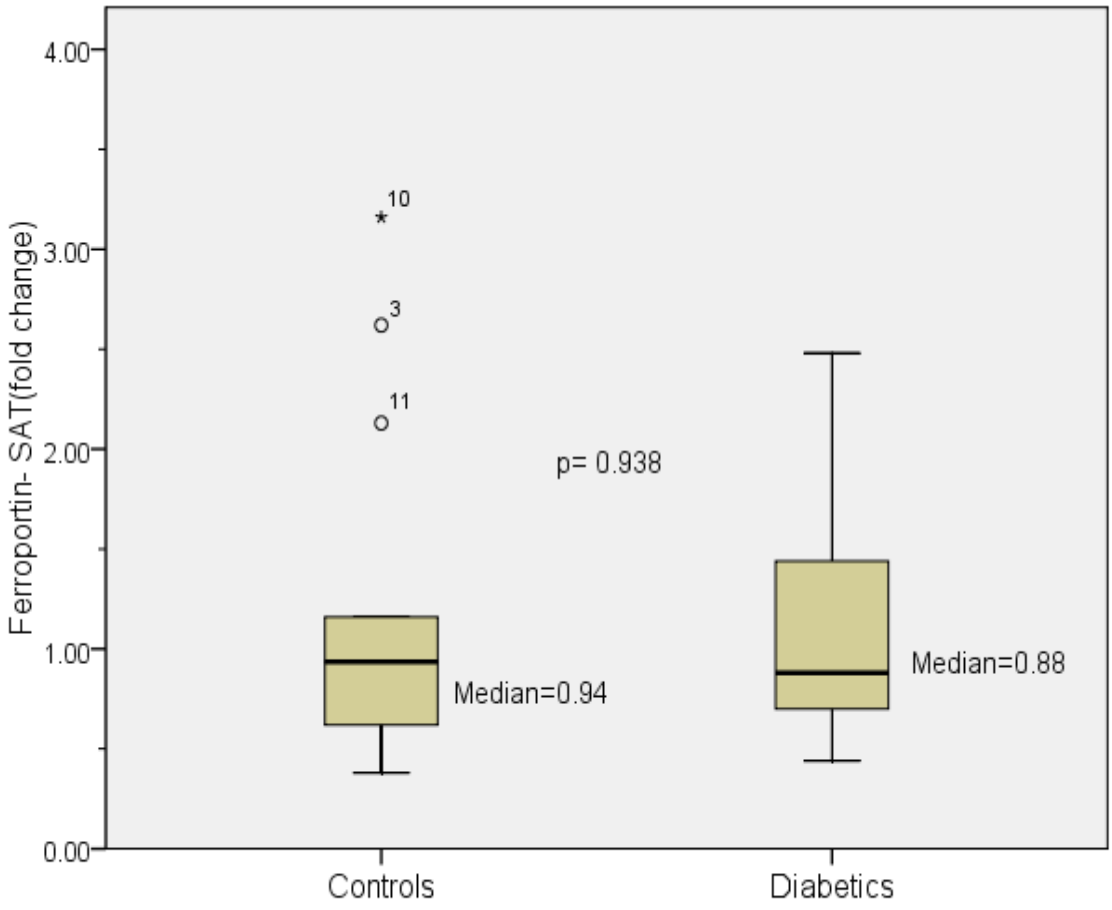
Figure 6.5. Relative mRNA expression of TfR1 in subcutaneous adipose tissue (SAT) in control and diabetics subjects



Data are shown as box and whisker plots, with medians and quartiles shown.

The relative mRNA expression of TfR1 in subcutaneous adipose tissue (SAT) in control and diabetic subjects were similar.

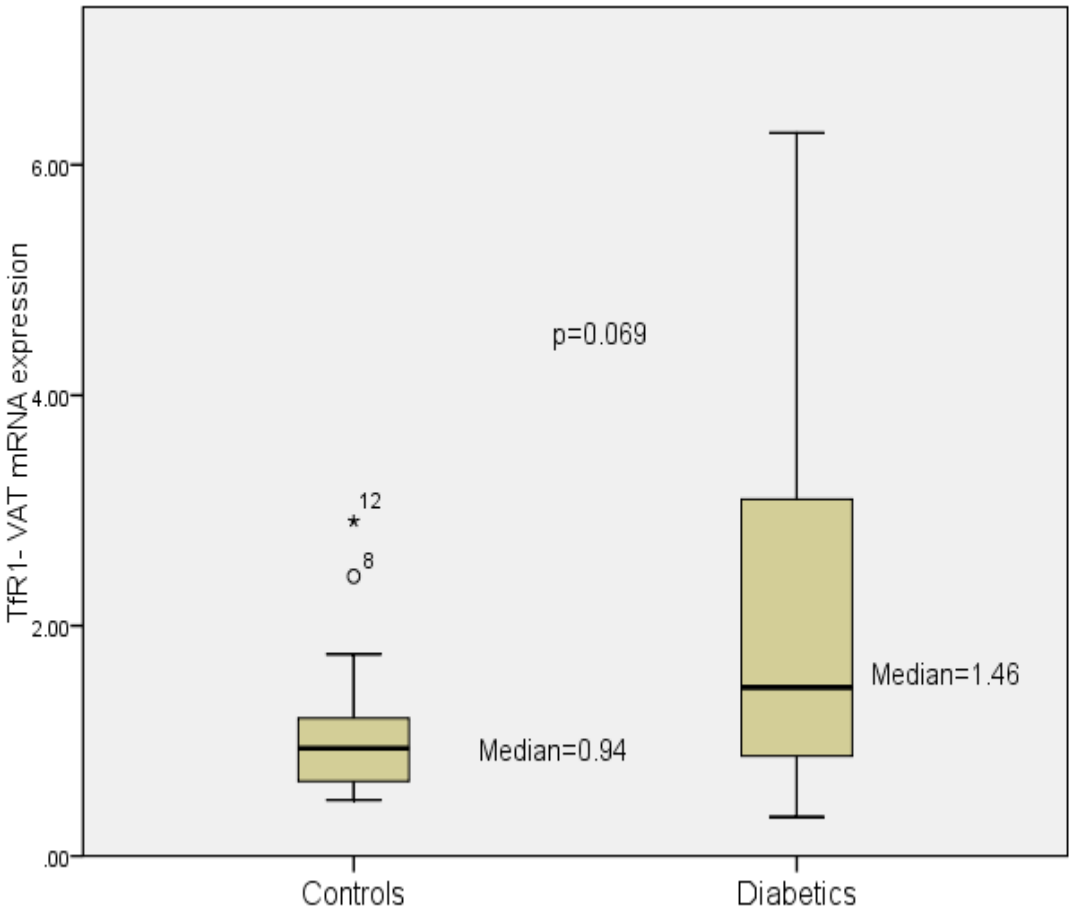
Figure 6.6. Relative mRNA expression of ferroportin in SAT in control and diabetic subjects



Data are shown as box and whisker plots, with medians and quartiles shown.

Relative mRNA expression of ferroportin in SAT was similar in the two groups.

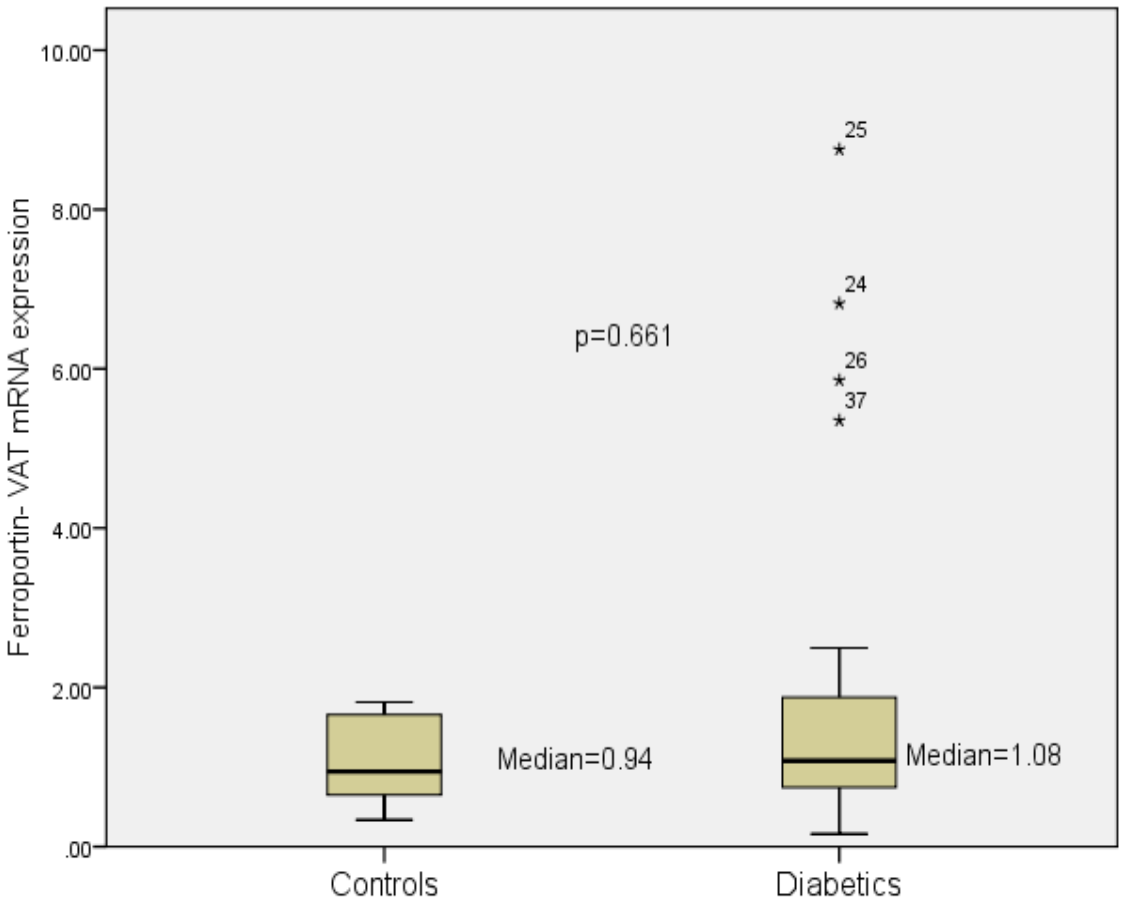
Figure 6.7. Relative mRNA expression of TfR1 in visceral adipose tissue (VAT) in control and diabetics



Data are shown as box and whisker plots, with medians and quartiles shown.

Relative mRNA expression of TfR1 in visceral adipose tissue (VAT) tended to be higher in the diabetics, compared to controls ($p = 0.069$).

Figure 6.8. Relative mRNA expression of ferroportin in VAT in diabetics and controls



Data are shown as box and whisker plots, with medians and quartiles shown.

Relative mRNA expression of ferroportin in VAT was found to be similar in both groups.

Correlation analysis

Correlation analysis was done on the data obtained.

Table 6.3. Correlation analysis of general parameters

Parameters		**Hb**	**Creatinine**	**HbA1c**
BMI	r	-.085	-.461	.087
	p	.617	.004	.634
Hb	r	-	.204	.040
	p	-	.226	.828
Creatinine	r	.204	-	-.130
	p	.226	-	.479
HbA1c	r	.040	-.130	-
	p	.828	.479	-

r- Spearman correlation coefficient

There was a significant positive correlation between BMI and serum creatinine among the subjects in the study.

Table 6.4. Correlation analysis of general parameters with serum and adipose tissue iron parameters

Parameters		**Iron**	**Ferritin**	**TIBC**	**Tfsat**	**TfR1 SAT**	**Fpn SAT**	**TfR1 VAT**	**Fpn SAT**
BMI	r	-.110	-.193	.233	-.163	.311	.177	.267	.106
	p	.515	.253	.165	.335	.061	.295	.110	.533
Hb	r	.378	.143	-.170	.408	-.230	.020	-.171	-.273
	p	.021	.399	.314	.012	.170	.908	.312	.102
Creatin -ine	r	.130	.200	-.128	.180	-.490	-.194	-.395	-.213
	p	.442	.235	.451	.286	.002	.251	.016	.207
HbA1c	r	-.014	-.125	.359	-.055	.267	-.052	.348	.005
	p	.939	.496	.043	.763	.140	.776	.051	.980

r- Spearman correlation coefficient

A significant positive correlation was found between the following parameters:

Hemoglobin and iron

Hemoglobin and transferrin saturation

HbA1c and TIBC

A significant negative correlation was observed between the following parameters:

Creatinine and TfR1 mRNA in SAT and VAT

Table 6.5. Correlation analysis of serum iron parameters with serum and adipose tissue iron parameters

Parameter		Iron	Ferritin	TIBC	Tfsat	TfR1 SAT	Fpn SAT	TfR1 VAT	Fpn SAT
Iron	r	-	.537	.010	.948	-.234	-.007	-.243	.044
	p	-	.001	.952	<.001	.163	.967	.147	.795
Ferri-tin	r	.537	-	-.273	.545	-.478	-.058	-.247	.235
	p	.001	-	.103	<.001	.003	.733	.140	.162
TIBC	r	.010	-.273	-	-.227	.317	.017	.390	-.064
	p	.952	.103	-	.176	.056	.921	.017	.706
Tfsat	r	.948	.545	-.227	-	-.297	.031	-.300	.052
	p	.000	.000	.176	-	.074	.857	.072	.759

r- Spearman correlation coefficient

A significant positive correlation was found between the following parameters:

Iron and ferritin

Iron and Tfsat

Ferritin and Tfsat

TIBC and TfR1 mRNA expression in VAT

A significant negative correlation was found between the following parameter

Ferritin and TfR1 mRNA in SAT

Table 6.6. Correlation analyses of expression of iron-related proteins in adipose tissue

Parameters		TfR1 mRNA in SAT	Ferroportin mRNA in SAT	TfR1 mRNA in VAT	Fpn mRNA in VAT
TfR1 mRNA in SAT	r	-	.107	.598	.084
	p	-	.528	<.001	.622
Ferroportin mRNA in SAT	r	.107	-	.271	.484
	p	.528	-	.105	.002
TfR1 mRNA in VAT	r	.598	.271	-	.430
	p	.000	.105	-	.008
Fpn mRNA in VAT	r	.084	.484	.430	-
	p	.622	.002	.008	-

r- Spearman correlation coefficient

A significant positive correlation was found between the following parameters:

TfR1 mRNA in SAT and VAT

Ferroportin mRNA in SAT and VAT

TfR1 and ferroportin mRNA in VAT

Table 6.7. Correlation analyses of iron-related proteins in adipose tissue of control subjects

		TfR1 mRNA in SAT	**Ferroportin mRNA in SAT**	**TfR1 mRNA in VAT**	**Fpn mRNA in VAT**
TfR1 mRNA in SAT	r	-	-0.037	0.535	0.268
	p	-	- 0.899	.049	0.353
Ferroportin mRNA in SAT	r	-0.037	-	0.279	0.591
	p	- 0.899	-	0.334	0.026
TfR1 mRNA in VAT	r	0.535	0.279	-	0.459
	p	.049	0.334	-	0.098
Fpn mRNA in VAT	r	0.268	0.591	0.459	-
	p	0.353	0.026	0.098	-

r- Spearman correlation coefficient

Controls

A significant positive correlation was observed between mRNA levels of TfR1 and ferroportin in the subcutaneous and visceral adipose tissue from control subjects.

Table 6.8. Correlation analyses of iron-related proteins in adipose tissue in diabetics

		TfR1 mRNA in SAT	**Ferroportin mRNA in SAT**	**TfR1 mRNA in VAT**	**Fpn mRNA in VAT**
TfR1 mRNA in SAT	r	-	0.233	0.621	0.047
	p	-	0.285	.002	0.831
Ferroportin mRNA in SAT	r	0.233	-	0.389	0.332
	p	0.285	-	0.067	0.122
TfR1 mRNA in VAT	r	0.621	0.389	-	0.429
	p	.002	0.067	-	0.041
Fpn mRNA in VAT	r	0.047	0.332	0.429	-
	p	0.831	0.122	0.041	-

r- Spearman correlation coefficient

Diabetics

A significant positive correlation was observed between mRNA levels of TfR1 in the subcutaneous and visceral adipose tissue from diabetic subjects. A significant positive correlation was also seen observed between mRNA levels of ferroportin and TfR1 in the visceral adipose tissue from the diabetics.

SUMMARY OF FINDINGS

1. The diabetic subjects in the study were significantly older than the control subjects.
2. BMI and hemoglobin levels were similar in diabetics and control subjects.
3. The HbA1c level was significantly higher in the diabetic group than in the control subjects, while serum creatinine was significantly higher in the control group.
4. Markers of iron status in serum (iron, ferritin, transferrin saturation and TIBC) were similar in diabetics and control subjects.
5. TfR1 gene expression in VAT tended to be higher in the diabetic group, compared to the control group.
6. Expression of TfR1 in SAT and ferroportin in VAT and SAT were similar in both groups.
7. Correlational analysis of all the subjects in the study showed the following:

   A significant positive correlation was found between the following parameters

   a) BMI and serum creatinine
   b) Hemoglobin and iron
   c) Hemoglobin and transferrin saturation
   d) HbA1c and TIBC
   e) Iron and ferritin
   f) Iron and Tfsat
   g) Ferritin and Tfsat
   h) TIBC and TfR1 mRNA expression in VAT
   i) TfR1 mRNA in SAT and VAT
   j) Ferroportin mRNA in SAT and VAT
   k) TfR1 and ferroportin mRNA in VAT

   A significant negative correlation was found between the following parameters

   a) Creatinine and TfR1 mRNA in SAT and VAT
   b) Ferritin and TfR1 mRNA in SAT

8. Correlational analysis of the control group showed the following:
   a. A significant positive correlation was observed between mRNA levels of TfR1 and ferroportin in the subcutaneous and visceral adipose tissue

9. Correlational analysis of the diabetic group has showed the following:
    a. A significant positive correlation was observed between mRNA levels of TfR1 in the subcutaneous and visceral adipose tissue.
    b. A significant positive correlation was observed between TfR1 and ferroportin in VAT of diabetics.

DISCUSSION

Increased body iron stores have been reported to be associated with insulin resistance, which is the pathognomonic feature of T2DM (Fernández-Real et al., 2002b;.Moreno-Navarrete et al., 2014; Salonen et al., 1998). Recent reports have suggested that the iron content in adipose tissue may play a role in the pathogenesis of insulin resistance (Dongiovanni et al., 2013; Gabrielsen et al., 2012; Orr et al., 2014; Wlazlo et al., 2013). However, it is not known whether the iron content in adipose tissue in patients with T2DM is different from that in non-diabetics.

In the present study, the gene expression of iron-related proteins, TfR1 and ferroportin, was determined in subcutaneous (SAT) and visceral (VAT) adipose tissue from diabetic and non-diabetic subjects. Serum markers of iron status (serum iron, ferritin, TIBC, and transferrin saturation) were also estimated in these subjects. It was found that TfR1 mRNA tended to be higher in the VAT of diabetic subjects than in control subjects ($p = 0.069$). TfR1 expression is chiefly regulated by the IRE-IRP system (Hentze et al., 2010b). When intracellular iron levels are low, IRP is activated and binds to the 3'-IRE on mRNA for TfR1; this stabilizes the mRNA, resulting eventually in increased protein levels. On the other hand, when iron levels in the cell are high, IRP levels are low; hence, binding to IRE on the mRNA for TfR1does not occur, resulting in accelerated degradation of the TfR1 mRNA by cytosolic RNAase (Hentze et al., 2010b). Thus, an increase in TfR1 mRNA suggests low intracellular iron, and vice-versa. Such an observation in the present study suggests the possibility that iron levels may be lower in the VAT of diabetics than in control subjects. However, ferroportin mRNA levels in both SAT and VAT, and TfR1 in SAT were found to be similar in the 2 groups.

There is a limited amount of literature currently available on iron status in adipose tissue in humans. Moreno-Navarrete et al (2014) determined gene expression of transferrin receptor, ferroportin and ferritin in adipose tissue in four independent cohorts. These cohorts consisted of subjects who were lean, overweight, obese and obese with T2DM. The authors have reported that TfR1 mRNA expression in SAT and VAT were lower in obese diabetic patients, while gene expression of ferritin and ferroportin were higher. These observations have been interpreted to be indicative of increased iron levels in the adipose tissue. Weight loss in these subjects, following bariatric surgery, resulted in increased expression of TfR1 and decreased expression of ferritin and ferroportin in adipose tissue. These observations have been taken to indicate decreased iron levels in adipose tissue, in the post-operative state. These changes were associated with improved insulin sensitivity in these subjects. Pihan-Le Bars et al (2016) have also reported that expression of TfR1 mRNA was lower in SAT and VAT from obese patients than in non-obese subjects, once again suggesting increased intracellular iron content in the adipose tissue. Ferroportin gene expression in the visceral adipose tissue of the obese subjects in their study was found to be higher than that in non-obese controls subjects, an observation that is in keeping with intracellular iron overloading. Expression of ferroportin in subcutaneous adipose tissue was, however, similar in both the groups. In addition, Pihan-Le Bars et al (2016) have reported that TfR1 and adiponectin gene expression in both SAT and VAT were positively correlated. Based on this, they have postulated that increased intracellular iron may play a role in suppressing adiponectin expression in adipose tissue, resulting in development of insulin resistance. No differences were noted between their diabetic group obese subjects (n = 16) and the non-diabetic obese subjects (n=30), with regard to serum markers of iron status (ferritin, serum iron, transferrin, transferrin saturation, total iron-binding capacity and sTfR) and serum CRP levels (Pihan-Le Bars et al., 2016).

It is difficult to make direct comparisons between these reported observations and the findings in the present study, as the subjects in the published studies described above were morbidly obese and underwent bariatric surgery, while none of the subjects in the present were obese. Hence, it is inevitable that findings in these 2 studies differ would differ from those of the present one.

Results of correlational analyses in the present study showed that gene expression levels of TfR1 and ferroportin in VAT of diabetics were significantly and positively correlated (Spearman's rho 0.429, p = 0.041). No such association was seen in the control group. This suggests that, in diabetics, iron stores in the adipocytes may be depleted as a consequence of increased expression of ferroportin, which is involved in export of iron from cells. It is possible that the resultant low intracellular levels of iron may have resulted in increased expression of TfR1.

In the present study, TfR1 mRNA levels in SAT were not significantly different in diabetics and control subjects (Fig.6.5); however, it was found to be positively correlated with TfR1 expression in the VAT in both diabetics and control subjects (Table 6.7 and 6.8). Similarly, ferroportin mRNA levels in SAT and VAT were positively correlated in control subjects (Spearman's rho 0.591, p=0.026) and diabetics (Spearman's rho 0.332, p=0.122). This suggests that, overall, changes in mRNA expression of TfR1 and ferroportin were similar in the SAT and VAT.

Interactions between obesity, diabetes and iron metabolism are complex. Obesity is an important risk factor for diabetes mellitus (Greenberg and Obin, 2006; Kasper et al., 2015). It has been shown that iron deficiency anemia is commonly associated with obesity (Amato et al., 2010; Pinhas-Hamiel et al., 2003; Wenzel et al., 1962; Yanoff et al., 2007). On the other

hand, iron overload has been reported to be associated with diabetes mellitus (Fernández-Real et al., 2002a; Ford and Cogswell, 1999; Forouhi et al., 2007). Adipose tissue metabolism has been shown to be affected by both iron deficiency and excess. In mice, where ferroportin was knocked-out specifically in adipocytes (resulting in increased iron content in the adipose tissue), insulin resistance and fasting hyperglycemia were seen as consequences (Gabrielsen et al., 2012). Mice fed a high-iron diet were found to develop iron overload in the VAT. This was associated with impaired insulin action in the adipose tissue (Dongiovanni et al., 2013). Iron deficiency, on the other hand, has been shown to impair adipogenesis (Moreno-Navarrete et al., 2014).

In the present study, both controls and diabetics were overweight, but not obese. About 90% of type 2 diabetics have been reported to be overweight or obese (Bray and Perreault, 2017). However, India has a high prevalence of lean T2DM (Barma et al., 2011), with 60% of T2DM being reported to be non-obese (Mohan et al., 1997). Indians are more prone develop a centripetal distribution of fat; they tend to have more VAT compared to Caucasians with the same BMI (Thomas et al., 2016). Dual energy X-ray absorptiometry (DEXA) measurements have shown that Asian Indian men have 7% higher percentage of body fat compared to Europeans with the same BMI in the same population (Thomas et al., 2016). In view of this, waist circumference (which is a direct indicator of intra-abdominal fat) is considered to be a better index of adiposity than BMI (Bray and Perreault, 2017; Thomas et al., 2016). In the present study, waist circumference was one of the anthropometric measurements carried out. However, 22 out of 37 subjects included in the study had large hernias, mostly large incisional hernias, which made it difficult to determine accurate values for waist circumference in these subjects.

Since there were no previous studies of this nature that provided information that could be used to calculate a sample size for this study (as discussed in the methodology section), it was decided to carry out a pilot study with 20 patients in each group. According to the design of the current study, the control subjects were supposed to be age-matched with the diabetics. During the period of the study (1 year), all consecutive subjects who were eligible (as per the inclusion and exclusion criteria of the study) were recruited. However, during the actual conduct of the study, it was found that it was not possible to meet the target of 20 patients in the control group, within the time available to carry out the work for this dissertation. This was because many of the subjects who were recruited as non-diabetics or control subjects were later found to have HbA1c levels that were indicative of a pre-diabetic state. Such subjects were not included as control subjects for the purposes of this study. Since the inclusion and exclusion criteria set out in the study were strictly adhered to, it was found that there were only 14 patients recruited who qualified to be control subjects. This was disappointing; however, it was decided to maintain the robustness of the design of the study by strict adherence to the inclusion and exclusion criteria used.

Age is an independent risk factor for the development of T2DM; older people are more affected by T2DM rather than young people (Kasper et al., 2015). Hence, it was not surprising to find that the mean age of the diabetics was significantly higher than the mean age of control subjects. Hence, age-matching was not achieved in the present study. Multiple linear regression analysis will be required to elucidate the effect of age on the outcomes measured; however, this was not done at this point, as the sample size was small. The study is being continued to attempt to achieve adequate sample size for the study and to allow multivariate analysis to be carried out at a later time point.

The mean values for serum creatinine in both groups were within the reference range. However, levels in the control group were found to be significantly higher than in the diabetics. In addition, a significant positive correlation was seen between BMI and serum creatinine levels among the subjects. The reason for these observations is currently unclear. The data obtained from a larger sample size will be examined carefully to see if this trend continues to be seen.

Among the 24 females who were included in the study, 16 were post-menopausal. Interestingly, 14 out of the 16 were in the diabetic group and only 2 in the control group. This finding is in accordance with the previous studies done worldwide stating the vulnerability of post-menopausal women to development of insulin resistance and metabolic syndrome (Jouyandeh et al., 2013; Marchi et al., 2017; Pandey et al., 2010).

In summary, the results of the present study showed that values for blood markers of iron status were similar in diabetics and non-diabetics. Gene expression of TfR1 and ferroportin were similar in the sub-cutaneous adipose tissue from both groups. In the visceral adipose tissue from diabetics, TfR1 mRNA levels tended to be higher than in control subjects; ferroportin expression levels were similar. This increased expression of TfR1 mRNA suggests that intracellular iron levels in adipose tissue were low. TfR1 and ferroportin mRNA levels were found to be positively correlated with one another in visceral adipose tissue of the diabetic group. These findings needed to be corroborated by studying an adequate number of patients, and by determining serum hepcidin levels and the iron content of adipose tissue. Such studies are on-going in the research group in the department that works on these aspects, in an attempt to understand better the complex interactions between iron metabolism in the adipose tissue, and insulin resistance.

CONCLUSION

The observations of this study suggest that adipocytes in VAT from diabetics may be iron-depleted, as indicated by a trend for TfR1 mRNA levels to be higher in diabetics.
This preliminary finding needs to be validated by studying an adequate number of age-matched patients, along with determination of the iron content in the adipose tissue and other iron-related parameters.

LIMITATIONS OF THE STUDY

1. The number of patients in the study group was small - 23 patients with T2DM and 14 control subjects.
2. Attempts were made to match diabetics and control subjects with regard to age, gender and BMI. However, it was found that the number of control subjects who satisfied the inclusion and exclusion criteria of the study were limited. Hence, it was not possible at this point to achieve the intended matching of diabetics and control subjects.
3. It was not possible to get accurate measurements of waist circumference (as an indicator of central obesity) in the subjects of the study, due to the large incisional hernias that many of them had.
4. Data on levels of serum hepcidin and inflammatory markers and iron content of the adipose tissue would have contributed to determining the true iron status of the patients in the study. However, it was not possible to carry out assays for these parameters, due to financial constraints.

FUTURE DIRECTIONS

Continuation of the present study, to achieve adequate sample size and involving study of more parameters relevant to iron homeostasis, is required to better understand how iron metabolism in adipose tissue is affected in patients with diabetes mellitus. This is currently on-going.

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APPENDIX I: LETTER OF APPROVAL FROM THE IRB

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

June 07, 2016

Dr. Rosa Mariam Mathew,
PG Registrar,
Department of Biochemistry,
Christian Medical College,
Vellore 632 004.

Sub: **Fluid Research Funding: New Proposal**
Iron-related parameters in adipose tissue and blood in diabetes mellitus
Dr. Rosa Mariam Mathew, Employment Number: 21194 , PG Registrar, Department of Biochemistry, Dr. Molly Jacob, Employment Number: 14509, Biochemistry, Dr. Joe Varghese, Employment no: 20405, Biochemistry, Dr. Inian S, Professor, Department of Surgery, Unit 3, Ms.Thenmozhi Mani, Biostatistics

Ref: IRB Min No: 9902 [OBSERVE] dated 05.02.2016

Dear Dr. Rosa Mariam Mathew,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Biju George, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS ... DM
SECRETARY (E... MMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Molly Jacob, Professor, Department of Biochemistry, CMC

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
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Ref: IRB Min No: 9902 [OBSERVE] dated 05.02.2016

Dear Dr. Rosa Mariam Mathew,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Iron-related parameters in adipose tissue and blood in diabetes mellitus" on February 05th 2016.

The Committee reviewed the following documents:

1. IRB Application format
2. Proforma
3. Patient Information Sheet and Informed Consent Form (English, Tamil)
4. Cvs of Drs. Molly Jacob, Joe Varghese, . Inian S, Ms.Thenmozhi Mani., Rosa Mariam Mathew
5. No. of documents 1 - 4

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on February 05th 2016 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, Research), Additional Vice Principal , Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC,Vellore	Internal, Clinician
Dr. Nihal Thomas	MD, MNAMS, DNB (Endo), FRACP (Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. CMC, Vellore	Internal, Clinician
Dr. Jayaprakash Muliyil	BSc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, Vellore	External, Scientist &Epidemiologist
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Mrs. Sheela Durai	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. B. J. Prashantham	MA(Counseling Psychol MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centr Vellore	External, Social Scientist
Dr. RatnaPrabha	MBBS, MD (Pharma)	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Dr. Vivek Mathew	MD (Gen. Med.) DM (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC, Vellore	Internal, Clinician
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Inian Samarasam	MS, FRCS, FRACS	Professor, Surgery, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "Iron-related parameters in adipose tissue and blood in diabetes mellitus" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in)

Fluid Grant Allocation:

A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty thousand only) each will be released at the end of the first year as 2 nd Installment.

Yours sincerely

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
M.D., D.M.
SECRETARY (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min No: 9902 [OBSERVE] dated 05.02.2016

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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APPENDIX II: INFORMATION SHEET FOR PATIENTS

Departments of Biochemistry and Surgery (Unit 3 and Unit 4)

Christian Medical College, Vellore

Iron-related parameters in adipose tissue and blood in diabetes mellitus

The Department of Biochemistry at Christian Medical College, Vellore, in association with the Department of Surgery, Unit 3, is carrying out a study to understand the relationship between diabetes mellitus (in which condition the action of insulin in the body is less effective) and iron in fat tissue. In order to do this study, we will need some fat tissue from under the skin over your abdomen and also from inside your abdomen. We request permission to take this tissue when your abdomen is opened during the surgery that you are going to have. There will be no increased risk to you on account of this. We would also like to ask if you are willing to allow us to measure your height, weight, and waist and hip circumferences and to provide 10 ml of blood the day before the surgery for measuring certain substances in the blood, related to diabetes and iron. Collection of this sample of blood will not cause harm to your health in any foreseeable manner.

You may not directly benefit from the study. However, if you are willing to participate in the study and provide the samples requested, it will help us to better understand the relationship between diabetes mellitus and iron. This knowledge may help doctors find ways to prevent or delay the onset of diabetes mellitus.

The blood and fat tissue samples collected will be used only for research purposes. You will not incur any expenses on account of the tests that will be done on your samples. If there is any sample remaining after this study is completed, we would like to request you for permission to store these and use them for future similar studies. All your medical information will be kept confidential.

The results of this study will be published in future in a medical journal, but you will not be identified by name in any publication or presentation of results. However, your medical notes may be reviewed by people associated with the study, without your additional permission. .

If you do not wish to give the blood and fat tissue samples requested, you are free to say so. It will not affect the treatment you will receive in the hospital. If you have any further queries, please contact one of us on the numbers provided below.

Dr. Rosa Mariam Mathew, Department of Biochemistry, CMC, Bagayam, Vellore, 632002
Contact number- 7358886835; Email id- leyzmathew@gmail.com

Dr. Inian S, Professor, Department of Surgery, Unit 3, CMC,
Vellore 632004. Contact number- 0416 2283054

Dr. Sukria Nayak, Department of Surgery, Unit 4, CMC,
Vellore 632004

Dr Molly Jacob, Professor, Department of Biochemistry, CMC, Bagayam, Vellore -632002
Contact number- 0416 2284267

INFORMED CONSENT FORM

The doctor has explained to me the details of the study proposed. I have understood what has been said, including the following:

1. If I agree to participate in this study, my height and weight will be measured and a blood sample (6 ml) will be collected from me from me the day before I have my surgery.

2. Samples of fat tissue will be collected from under the skin of my abdomen and also from inside my abdomen, during the surgery.

3. Giving these samples will not affect my health in any foreseeable way.

4. The samples taken will be used only for research purposes. If there are any samples remaining after this study is completed, they will be stored and may be used for future related studies.

5. I understand that my identity will not be revealed in any information released to third parties or published or in any presentation at scientific meetings.

6. I voluntarily agree to take part in this study and donate samples of fat tissue and 10 ml of blood.

7. I have had the opportunity to ask questions to clarify my doubts.

Signature/thumb print of participant: Signature of investigator
Name of the participant:
Date:

Signature/thumb print of witness:
Relationship to participant
Date:

Name of the person who has taken the informed consent:

Signature:

APPENDIX III: PATIENT PROFORMA

DEPARTMENT OF BIOCHEMISTRY AND SURGERY
CHRISTIAN MEDICAL COLLEGE, VELLORE- 632002

Iron-related parameters in adipose tissue and blood in diabetes mellitus

PROFORMA FOR DETAILS OF PARTICIPANTS

Name	Age
Sex	Hospital No
Address	Date

1. Personal history:

Height: cm Weight: kg BMI: kg/m^2

Alcohol use: Yes/ No. If yes, no. of years: Qty consumed: per day/per week

Smoking: Yes/ No. If yes, no of years: Qty consumed: per day/per week

Any history of recent blood donation/ blood transfusion: Yes/ No

Only for females
Attained menopause: Yes/No
If not, do you have regular periods: Yes/No

2. Medical history:
H/o of any recent infection: Yes/ No. If yes, specify nature of infection and treatment:

H/o diabetes: Yes/No. If yes no of years since diagnosis

Current treatment

H/o renal disease: Yes/No

H/o liver disease: Yes/No

H/o of any malignancy: Yes/No.

3. Treatment history:
Are you any other medications: Yes/No. If yes, give details

4. Diagnosis:

Type of surgery posted for:

Indication for surgery:

5. Laboratory investigations

Hemoglobin:	TIBC
Serum iron:	Serum creatinine:
Serum ferritin:	Transferrin saturation:
HBA_{1c}	

Appendix 4: Master sheet with data

Controls

S.No	Sex	Age	BMI	Hb	Creat	HbA1c	Iron	Ferritin	UIBC	TIBC	T sat	SAT TfR1	SAT Fpn	VAT Tfr1	VAT Fpn
1	M	31	28.2	11.5	0.73	5.5	51	120	230	281	18.15	1.91	0.90	0.91	1.82
2	M	40	22.9	12.8	0.91	5.1	67	177.6	276	343	19.53	0.40	0.62	0.54	0.50
3	M	47	24.1	13.9	0.83	5.5	93	43.8	179	272	34.19	0.48	2.62	0.69	1.79
4	M	45	19.5	11.7	0.82	5	33	24.2	238	271	12.18	1.41	0.52	1.03	0.64
5	M	48	22.6	12.8	0.99	5	16	66.2	252	268	5.97	0.38	0.38	0.59	0.34
6	F	27	35.2	13	0.65	4.6	82	111.3	233	315	26.03	0.67	0.97	0.49	0.90
7	F	33	34	14.5	0.65	5.4	93	54.7	205	298	31.21	1.57	1.16	0.65	0.65
8	F	36	34	10.5	0.57	5.4	29	7.8	331	360	8.06	1.74	0.86	2.43	1.66
9	F	42	34.4	12.5	0.68	5.4	56	59.8	247	303	18.48	1.51	0.81	0.90	0.85
10	F	47	33.8	13.1	0.65	5.3	44	54.1	268	312	14.10	1.11	3.16	1.75	1.46
11	F	45	25.4	12.9	0.79	5.1	47	123	201	248	18.95	0.61	2.13	1.09	1.74
12	F	50	35.6	12.1	0.75	5.6	24	6.3	386	410	5.85	2.00	0.99	2.91	0.90
13	F	51	21.5	12.4	0.77	5.3	73	28.7	299	372	19.62	1.51	0.56	0.96	0.99
14	F	47	30.9	11.4	0.73	5.7	74	56.4	185	259	28.57	0.92	1.14	1.20	1.54

Diabetics

S.No	Sex	Age	BMI	Hb	Creat	HbA1c	Iron	Ferritin	UIBC	TIBC	T sat	SAT TfR1	SAT Fpn	VAT Tfr1	VAT Fpn
15	M	61	26.8	14.6	0.78	11.6	72	135.2	289	361	19.94	0.54	0.59	0.81	0.16
16	M	44	27.1	16.7	0.7		165	144.2	97	262	62.98	0.48	0.63	0.66	0.93
17	M	47	24.8	14.7	0.8	8.3	91	33.3	275	366	24.86	0.78	0.88	0.94	0.55
18	M	42	26.8	13.2	0.97	9.1	25	9	393	418	5.98	0.67	1.67	1.21	0.92
19	M	46	26.8	15.3	0.69	7.3	81	29.8	242	323	25.08	0.98	1.33	0.93	0.82
20	M	60	19.8	11.7	0.94	8.7	39	115	180	219	17.81	0.54	2.03	0.37	1.14
21	M	59	24	13.6	0.76	10.4	59	29	245	304	19.41	3.61	0.67	1.67	0.84
22	M	53	27.6	13.5	0.64	7.4	63	56.6	241	304	20.72	0.80	0.74	4.35	1.08
23	F	60	25.9	11.5	0.64		67	123	445	512	13.09	1.34	0.73	2.46	1.58
24	F	47	32.9	11.2	0.72	9.7	32	43.1	289	321	9.97	1.01	0.76	5.70	6.82
25	F	56	34.2	13.8	0.56		50	114	249	299	16.72	1.47	1.39	3.39	8.75
26	F	48	22.5	10.6	0.58	5.2	55	89	246	301	18.27	0.38	1.31	0.34	5.86
27	F	44	28.4	11.8	0.89	9.5	86	111	300	386	22.28	1.48	0.44	1.07	1.10
28	F	49	28.1	14.2	0.6	7.6	53	75.2	240	293	18.09	1.34	0.81	1.46	1.09
29	F	52	42.3	12.1	0.59	7.8	31	21.4	249	280	11.07	0.78	0.61	0.73	0.63
30	F	47	31.1	13	0.5	9.4	22	29.8	283	305	7.21	2.38	0.91	1.72	1.26
31	F	48	26	12.9	0.54		42	23.5	278	320	13.13	3.63	1.72	6.15	2.50
32	F	50	32.8	11.8	0.6	6.6	39	13.6	392	431	9.05	2.66	2.48	2.81	0.91
33	F	46	32.8	11.6	0.41	9.9	45	27	322	367	12.26	1.46	0.63	0.70	0.67
34	F	47	21.6	12.4	0.55		43	33.1	193	236	18.22	2.06	0.75	1.45	0.52
35	F	57	27.7	11.7	0.66	9.6	77	122	294	371	20.75	1.72	2.04	6.28	2.17
36	F	53	32.9	10.6	0.47	8.4	42	12	382	424	9.91	2.20	1.47	1.84	0.51
37	F	60	29	12	0.55	6.7	87	277.7	236	323	26.93	1.34	1.41	3.78	5.35

APPENDIX V: MIQE CHECKLIST FOR qPCR

Item to check	Importance *	Response
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	E	Subcutaneous and visceral adipose tissue obtained from diabetics and controls were used for all the experiments
Number within each group	E	In all experiments, 23 diabetic patients and 14 controls were used.
Assay carried out by core lab or investigator's lab?	D	Assays were carried out in the investigators' lab
SAMPLE		
Description	E	Subcutaneous and visceral adipose tissue
Volume/mass of sample processed	D	Small piece of the adipose tissue samples
Microdissection or macrodissection	E	Not applicable
Processing procedure	E	0.8 mL of Tri-reagent was added to one eppendorf tube in which the adipose tissue sample was collected.
If frozen - how and how quickly?	E	Homogenized samples were immediately transferred to a -70°C freezer
If fixed - with what, how quickly?	E	Not applicable
Sample storage conditions and duration (especially for FFPE samples)	E	Adipose tissue samples in plain microtube were snap frozen in liquid nitrogen immediately and transferred to a -70°C freezer
NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	E	Guanidinium thiocyanate-phenol-chloroform extraction method using Tri-reagent (Sigma)
Name of kit and details of any modifications	E	Not applicable
Source of additional reagents used	D	Chloroform and isopropanol used for RNA isolation were of molecular biology grade obtained from Sigma.
Details of DNase or RNAse treatment	E	DNAase treatment using the Ambion TURBO DNA-free kit was done.
Contamination assessment (DNA or RNA)	E	All samples were run on 1% agarose gel to look for DNA contamination and RNA integrity.
Nucleic acid quantification	E	Done using a nanospectrophotometer
Instrument and method	E	NanoDrop2000c from Thermo Fischer
Purity (A260/A280)	D	A260/A280 for all samples were > 1.80
RNA integrity method/instrument	E	All samples were run on a 1% agarose gel. Only those samples that showed clear and distinct bands corresponding to 18s and 28s rRNA were used for cDNA construction

RIN/RQI or Cq of 3' and 5' transcripts	E	Not done
REVERSE TRANSCRIPTION		
Complete reaction conditions	E	For 1 reaction, 5x Prime script buffer 2µL, RT enzyme mix with RNase inhibitor 0.5 µL, Oligo dT primer 0.5 µL (final concentration 25pmol),Random 6mers 0.5 µL (final concentration 50pmol)
Amount of RNA and reaction volume	E	500ng of total RNA was added to a total volume of 10µL
Priming oligonucleotide (if using GSP) and concentration	E	Not applicable
Reverse transcriptase and concentration	E	PrimeScript™ RT enzyme mix (Concentration not specified by the kit manufacturer)
Temperature and time	E	37^0C for 15 minutes, 85^0C for 5seconds, 4^0 C for 10 minutes
Manufacturer of reagents and catalogue numbers	D	PrimeScript™ RT Reagent Kit (Perfect Real Time) TaKaRa Clontech. Catalog number RR037A
Storage conditions of cDNA	D	-20°C
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay	E	Not applicable
Sequence accession number	D	Information provided in Table
Amplicon length	E	Information provided in Table
In silico specificity screen (BLAST, etc)	E	Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to check the specificity of each primer-pair used.
Pseudogenes, retropseudogenes or other homologs?	D	No
Location of each primer by exon or intron (if applicable)	E	Not applicable
What splice variants are targeted?	E	Primers were designed to amplify all splice variants of the target genes
qPCR OLIGONUCLEOTIDES		
Primer sequences	E	Information provided in Table
RT Primer DB Identification Number	D	Not applicable
Probe sequences	D	Not applicable
Location and identity of any modifications	E	Not applicable
Manufacturer of	D	Beta- actin: Sigma, India

oligonucleotides		TfR1, FPN: Eurogentec, Belgium
qPCR PROTOCOL		
Reaction volume and amount of cDNA/DNA	E	10 µL reaction volume containing 2 µL cDNA diluted 1:10
Primer, (probe), Mg++ and dNTP concentrations	E	Final concentrations were: Primer: 250nM Mg^{2+} : 2.5mM dNTPs: not specified by the kit manufacturer
Polymerase identity and concentration	E	TaKaRa Ex Taq HS DNA Polymerase (concentration not specified by the kit manufacturer)
Buffer/kit identity and manufacturer	E	SYBR® Premix Ex Taq™ II (Tli RNaseH Plus)Cat # RR820A
Exact chemical constitution of the buffer	D	Information not provided by the kit manufacturer
Additives (SYBR Green I, DMSO, etc.)	E	Not applicable
Manufacturer of plates/tubes and catalog number	D	96-well plates from Axygen Scientific (catalogue number: PCR-96-FS-C)
Complete thermocycling parameters	E	95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec)
Reaction setup (manual/robotic)	D	Manual
Manufacturer of qPCR instrument	E	BioRad Chromo4 real-time PCR machine
qPCR VALIDATION		
Specificity (gel, sequence, melt, or digest)	E	Melt curve analysis was done for all PCR runs for all the genes. Single peaks were detected. No primer dimers were seen in any of reaction wells.
For SYBR Green I, Cq of the NTC	E	Information provided in the table
Standard curves with slope and y-intercept	E	Information provided in the table
PCR efficiency calculated from slope	E	Information provided in the table
r2 of standard curve	E	Information provided in the table
Linear dynamic range	E	Information provided in the table
Cq variation at lower limit	E	Information provided in the table
If multiplex, efficiency and LOD of each assay.	E	Not applicable
DATA ANALYSIS		
qPCR analysis program (source, version)	E	MJ Opticon Monitor Analysis Software Version 3.1 (BioRad)
Cq method determination	E	Manual
Outlier identification and disposition	E	Not applicable

Results of NTCs	E	Information provided in the table
Justification of number and choice of reference genes	E	The reference gene used was beta-actin. The choice was based on previous publications which have used beta actin as the reference gene.
Description of normalisation method	E	The delta delta Ct method was used for normalization
Number and stage (RT or qPCR) of technical replicates	E	All reactions were conducted in duplicate when qPCR was carried out.
Repeatability (intra-assay variation)	E	Average of Cq values for duplicates was taken for calculation. Runs were repeated in samples where the Cq SD exceeded 0.2
Statistical methods for result significance	E	The Krushkal Wallis test was used to detect statistically significant changes occurring in the different groups of mice. Mann Whitney test was used for all pair-wise comparisons.
Software (source, version)	E	SPSS version 16.0

* E – essential, D - desirable